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(57) Abstract

Described are recombinant DNA molecules comprising the regulatory sequence(s) of an intron of the Endothelial Growth Factor (VEGF) receptor-2 gene (Flk-1) or of a gene homologous to the Flk-1 gene, being capable of conferring expression of a heterologous DNA sequence in endothelial cells, preferably in vivo. Vectors comprising said DNA molecules as well as host cells containing the same are provided. Also provided are pharmaceutical and diagnostic compositions comprising such recombinant DNA molecules and vectors. Furthermore, cells and transgenic non-human animals, comprising the aforementioned recombinant DNA molecules or vectors stably integrated into their genome and their use for the identification of substances capable of suppressing or activating transcription of a gene in endothelial cells are described. Described is further the use of the before described recombinant DNA molecules and vectors for the preparation of pharmaceutical compositions for treating, preventing, and/or delaying a vascular or tumorous disease in a subject. Furthermore, uses of the recombinant DNA molecules and vectors of the invention for the preparation of pharmaceutical compositions for inducing a vascular or tumorous disease in a non-human animal are provided.

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REGULATORY SEQUENCES CAPABLE OF CONFERRING EXPRESSION OF A HETEROLOGOUS DNA SEQUENCE IN ENDOTHELIAL CELLS IN VIVO AND USES THEREOF

The present invention relates to recombinant DNA molecules comprising the regulatory sequence(s) of an intron of the Vascular Endothelial Growth Factor (VEGF) receptor-2 gene (Flk-1) or of a gene homologous to the Flk-1 gene, being capable of conferring expression of a heterologous DNA sequence in endothelial cells in vivo. The present invention also relates to vectors comprising said recombinant DNA molecules as well as to host cells transformed with such recombinant DNA molecules or vectors. The present invention additionally relates to pharmaceutical and diagnostic compositions comprising such recombinant DNA molecules, vectors or cells. Furthermore, the present invention relates to cells and transgenic non-human animals, comprising the aforementioned recombinant DNA molecules or vectors stably integrated into their genome and their use for the identification of substances capable of suppressing or activating transcription of a gene in endothelial cells. The present invention also relates to the use of the before described recombinant DNA molecules and vectors for the preparation of pharmaceutical compositions for treating, preventing, and/or delaying a vascular or tumorous disease in a subject. Furthermore, the recombinant DNA molecules and vectors of the invention can be used for the preparation of pharmaceutical compositions for inducing a vascular or tumorous disease in a non-human animal.

In the field of neuroscience and medical therapy, there is a great demand for test systems to study the function and interaction of gene products, the malfunction or expression of which cause vascular and/or tumorous diseases. Such systems would

also be suitable for drug development against such diseases. A prominent example for gene products involved in vascular diseases are angiogenic growth factors and their endothelial receptors which play a major role in the formation of the embryonic vascular system and in certain angiogenesis-dependent diseases, such as solid tumor growth or retinopathy. The Kinase-insert Domain-containing Receptor/fetal liver kinase-1 (KDR/Flk-1) in the following referred to as Flk-1 and Flt-1 are high affinity signaling receptors for the endothelial mitogen, vascular endothelial growth factor (VEGF) (Connolly, J. Clin. Invest. 84 (1989), 1470-1478; Leung, Science 246 (1989), 1306-1309). Through interactions with its receptors, VEGF plays critical roles in growth and maintenance of vascular endothelial cells and in the development of new blood vessels in physiologic and pathologic states (Aiello, New Engl. J. Med. 331 (1994), 1480-1487; Shweiki, Nature 359 (1992), 843-845; Berkman, J. Clin. Invest. 91 (1993), 153-159). The patterns of embryonic expression of VEGF suggest that it is crucial for differentiation of endothelial cells from hemangioblasts and for development of blood vessels at all stages of growth (Jakeman, Endocrinology 133 (1993), 848-859; Breier, Development 114 (1992), 521-532). Among many potentially angiogenic factors, VEGF is the only one with patterns of expression, secretion, and activity that suggest a specific angiogenic function in normal development (Klagsbrun, Current Biology 3 (1993), 699-702).

High-affinity receptors for VEGF are found only on endothelial cells, and VEGF binding has been demonstrated on macro- and microvascular endothelial cells and in quiescent and proliferating endothelial cells (Jakeman, Endocrinology 133 (1993), 848-859; Jakeman, Clin. Invest. 89 (1992), 244-253). The Flk-1 and Flt-1 have been identified as candidate VEGF receptors by affinity cross-linking and competition-binding assays (de Vries, Science 255 (1992), 989-991; Millauer, Cell 72 (1993), 835-846; Terman, Biochem. Biophys. Res. Commun. 187 (1992), 1579-1586). These two receptor tyrosine kinases contain seven similar extracellular immunoglobulin domains and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert (de Vries, Science 255 (1992), 989-991; Matthews, Proc. Natl. Acad. Sci. U.S.A 88 (1991), 9026-9030; Terman, Oncogene 6 (1991), 1677-1683); they are expressed specifically by endothelial cells *in vivo* (Millauer,

Cell 72 (1993), 835-846; Peters, Proc. Natl. Acad. Sci. USA 90 (1993), 7533-7537; Yamaguchi, Development 118 (1993), 489-498). *In situ* hybridization in the developing mouse has demonstrated that Flk-1 is expressed in endothelial cells at all stages of development, as well as in the blood island in which endothelial cell precursors first appear (Millauer, Cell 72 (1993), 835-846). Flk-1 is a marker for endothelial cell precursors at their earliest stages of development (Yamaguchi, Development 118 (1993), 489-498).

The vascular endothelium is critical for physiologic responses including thrombosis and thrombolysis, lymphocyte and macrophage homing, modulation of the immune response, and regulation of vascular tone. The endothelium is also intimately involved in the pathogenesis of vascular diseases such as atherosclerosis (Ross, Nature 362 (1993), 801-809). Although a number of genes expressed in the endothelium have been characterized (Collins, J. Biol. Chem. 266 (1991), 2466-2473; Iademarco, J. Biol. Chem. 267 (1992), 16323-16329; Jahroudi, Mol. Cell. Biol. 14 (1994), 999-1008; Lee, J. Biol. Chem. 265 (1990), 10446-10450), expression of these genes is either not limited to vascular endothelium (e.g., the genes encoding von Willebrand factor, endothelin-1, vascular cell adhesion molecule-1), or is restricted to specific subpopulations of endothelial cells (e.g., the gene for endothelial-leukocyte adhesion molecule-1). Flk-1 (also known as VEGF-receptor 2) is expressed in endothelial cells during embryonic and postnatal development. The Flk-1 receptor is the first endothelial receptor to be expressed in endothelial cell precursors during embryonic vascular development. Gene targeting experiments in transgenic mice have demonstrated that this receptor is essential for endothelial cell differentiation (Shalaby, Nature 376 (1995), 62-66). Furthermore, in a variety of tumors, Flk-1 receptor expression is re-induced in the tumor vasculature, and it has been shown that signaling via the Flk-1 receptor is required for tumor vascularization and growth (Millauer, Nature 367 (1994), 576-579).

Recently, in vitro studies with the upstream region of the human Flk-1 gene (Patterson, J. Biol. Chem. (1995), 23111-23118) showed that DNA fragments located in the 5' flanking region of the human Flk-1 gene mediate expression of a reporter gene.

For studying all aspects of genes involved in vascular diseases such as atherosclerosis, however, the system described by Patterson, supra, suffers from several drawbacks. For example, promoter activity of the 5'-flanking region used by Patterson was also observed in cell types which do not express the Flk-1 gene naturally. Furthermore, the promoter fragment employed in Patterson, supra, was not shown to be expressed in vivo in its natural background. In order to specifically suppress or confer endothelium specific gene expression and for the development of endothelium specific drugs, however, one needs test systems which closely resemble the regulation of the Flk-1 expression in vivo since otherwise non-informative or even false positive results may be obtained.

Thus, the technical problem of the present invention is to provide means and methods that allow the modulation of gene expression specifically in endothelial cells in vivo, preferably at all stages of development.
The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a recombinant DNA molecule comprising:

(a) a first regulatory sequence of an intron of the Vascular Endothelial Growth factor (VEGF) receptor-2 (Flk-1) gene or of a gene homologous to the Flk-1 gene being capable of conferring expression in endothelial cells in vivo; and
(b) operatively linked thereto a heterologous DNA sequence.

In accordance with the present invention, a regulatory sequence driving the expression of a heterologous DNA sequence in substantially all endothelial cells in vivo, preferably at substantially all stages of development has been identified. Said regulatory sequence is suitable to direct the expression of a heterologous DNA sequence in the above-mentioned cells. The recombinant DNA molecule of the invention allows studying the function and interaction of proteins which are expressed in the endothelium of, for example, humans and the malfunction, and/or

unregulated expression of which is supposed to be the or a causative agent of vascular and tumorous diseases. Thus, the regulatory sequences of the invention are particularly suited and useful for the engineering of transgenic cells and non-human animals which can serve as a test system for the development of drugs for the treatment of vascular and tumorous diseases of endothelial origin.

In the context of the present invention the term "a first regulatory sequence of an intron of the Flk-1 gene" means a nucleotide sequence of the first intron of the murine Flk-1 gene including the regulatory sequences which are capable of conferring the specific expression of a heterologous DNA sequence in endothelial cells, preferably at all stages of development.
The high affinity receptor for vascular endothelial growth factor (VEGF), Flk-1, is the first endothelial receptor to be expressed in angioblast precursors, and its function is essential for the differentiation of the hemangioblastic lineage. In accordance with the present invention cis-acting regulatory elements of the murine Flk-1 gene have been identified that mediate endothelial specific expression of a reporter gene in transgenic mice. Sequences within the 5'-flanking region of the Flk-1 gene, in combination with sequences located in the first intron, specifically and reproducibly targeted transgene expression to endothelial cells of the embryonic vasculature. These sequences were capable of targeting expression of the heterologous DNA sequence to angioblasts during early stages of vascular development and also to the vasculature of postnatal mice. The regulatory sequences located in the first intron also functioned as an autonomous endothelium-specific enhancer when fused to the heterologous herpes simplex virus-thymidine kinase promoter. This Flk-1 intron enhancer contains several potential binding sites for transcription factors of the Ets and GATA families. Sequences of the Flk-1 promoter contributed to a strong, complete and reproducible endothelial cell-specific gene expression in the embryo and are essential for expression in the yolk sac.
In order to characterize cis-acting regulatory sequences contained in the Flk-1 gene, recombinant bacteriophage lambda clones containing mouse genomic DNA (Mouse strain 129/Sv) have been isolated encompassing a 21 kilo base pair (kb) region of

the mouse Flk-1 gene, contained in the DNA insertions of λ phages 6 and 16, including approximately 15 kb of 5' flanking sequences, exons 1, 2 and 3, and introns 1 and 2 (Fig. 4A). The DNA sequence of a 12.8 kb region spanning from position -6.65 kb (the affixes - and + refer to the nucleotide position relative to the transcriptional start site as shown in Fig. 1 which corresponds to nucleotide position 6661 of SEQ ID NO: 1) to position +6.15 (located in the third exon) was determined (SEQ ID NO: 1). Reporter gene studies were performed in order to characterize regulatory cis-acting elements of the Flk-1 gene. Initial studies focused on the role of 5' flanking sequences of the Flk-1 gene ("Flk-1 promoter") in mediating endothelium-specific expression in cultured bovine aortic endothelial (BAE) cells (Rönicke, Circulation Research 79 (1996), 277-285). In these studies, it was found that a promoter fragment ranging from -624 to +299 mediated high expression of the luciferase reporter gene following transient transfection in BAE cells. Experiments with transgenic mouse embryos performed in accordance with the present invention revealed, however, that the murine promoter DNA fragments were not sufficient to mediate endothelium-specific reporter gene expression in vivo. Surprisingly, however, when a Flk-1 promoter fragment (ranging from, e.g., -624 to +299 bp) was combined with a 2.3 kb fragment of the first Flk-1 intron, endothelium specific expression of a lacZ reporter gene in mouse embryos was obtained. Thus, the first intron (nucleotides 7027 to 10642 of SEQ ID NO: 1) of the mouse Flk-1 gene is essential for endothelium specific gene expression. In particular, a DNA fragment (see Figure 12) comprising nucleotides 10094 to 10608 of SEQ ID NO: 1 was shown in accordance with the present invention to be sufficient to direct the expression of a heterologous DNA sequence into endothelial cells; see Example 8. This is a novel finding because the sequences described in previous publications (Patterson, supra; Rönicke, supra) are, in contrast to the expectations and interpretations of the prior art, not sufficient to mediate endothelium-specific expression in vivo. These results obtained in accordance with the present invention demonstrate that the regulatory sequences located in the intron of the Flk-1 gene mediating endothelium-specific expression can be used to direct expression of heterologous genes in the vasculature.

The genomic DNA of the murine Flk-1 gene comprising the intron regulatory sequences can be obtained from liver of mouse strain 129/SV, or, for example, by screening a phage library of liver genomic DNA in the vector λFixII (Stratagene, La Jolla, CA) generated by conventional methods known in the art.

The term "regulatory sequence of a gene homologous to the Flk-1 gene" also includes promoter regions and regulatory sequences of a gene from another species, for example, humans and other mammals which is homologous to the Flk-1 gene of mouse and which confers the same or substantially the same expression pattern. Such regulatory sequences are characterized by their capability of conferring expression of a heterologous DNA sequence specifically in endothelial cells in vivo, preferably at all stages of development. Thus, according to the present invention, regulatory sequences from other species can be used that are functionally homologous to the regulatory sequences of the intron of the Flk-1 gene from mouse, or regulatory sequences of genes that display a substantially identical pattern of expression, in the sense of being expressed in the endothelium, preferably at all stages of development.

It is possible for the person skilled in the art to isolate by employing the known Flk-1 gene from mouse, corresponding genes from other species, for example, humans and other mammals. This can be done by conventional techniques known in the art, for example, by using Flk-1 gene sequences as a hybridization probe or by designing appropriate PCR primers. It is then possible to isolate the corresponding regulatory sequences by conventional techniques and test them for their expression pattern. For this purpose, it is, for instance, possible to fuse the regulatory sequences to a reporter gene, such as the luciferase or green fluorescent protein (GFP) encoding genes and assess the expression of the reporter gene in transgenic animals, for example in mice. The partial nucleotide sequence of the human Flk-1 gene may be obtained from Genbank Acc. No. X89776; Patterson, supra; Terman, Biochem. Biophys. Res. Comm. 187 (1992), 1579-1586; Genbank Acc. No. X61656.

The present invention also relates to recombinant DNA molecules comprising regulatory sequences which are substantially identical to that of the Flk-1 intron or to

an intron of a homologous gene or to fragments thereof and which are able to confer specific expression in endothelial cells, preferably at all stages of development in mouse or other mammals.

Such regulatory sequences differ at one or more positions from the above-mentioned regulatory sequences but still have the same specificity, namely they comprise the same or similar sequence motifs responsible for the above described expression pattern. Preferably such regulatory sequences hybridize to one of the above-mentioned regulatory sequences, most preferably under stringent conditions. Particularly preferred are regulatory sequences which share at least 85%, more preferably 90-95%, and most preferably 96-99% sequence identity with one of the above-mentioned regulatory sequences and have the same specificity. Such regulatory sequences also comprise those which are analogues or derivatives, for example by way of nucleotide deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination in comparison to the above-described nucleotide sequence. Methods for introducing such modifications in the nucleotide sequence of the regulatory sequences of the invention are well known to the person skilled in the art and described, for example, in Sambrook (Molecular cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989)). All such fragments, analogues and derivatives of the regulatory sequence of the invention are included within the scope of the present invention, as long as the essential characteristic regulatory properties as defined above remain unaffected in kind. It is also immediately evident to the person skilled in the art that further regulatory sequences may be added to the regulatory sequences of the invention. For example promoters, transcriptional enhancers and/or sequences which allow for induced expression of the regulatory sequences of the invention may be employed. A suitable inducible system is for example tetracycline-regulated gene expression which is described by, e.g., Gossen (Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551; Trends Biotech. 12 (1994), 58-62).

The expression conferred by the regulatory sequences of the invention may not be exclusively limited to the above-described specificity but may also occur in, e.g.,

neuronal cells, including neural retinal progenitor cells at all or different stages of development and haematopoietic cells (Yang, J. Neurosci. 16 (1996), 6089-6099).

The term "further regulatory sequences" refers to sequences which influence the specificity and/or level of expression, for example in the sense that they confer cell and/or tissue specificity or developmentally and/or inducible regulated gene expression. Such regions can be located upstream of or comprising the transcription initiation site, such as a promoter, but can also be located downstream of it, e.g., in transcribed but not translated leader sequences.

The term "promoter" refers to the nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding, and also includes, for example, the TATA box.

The term "in vivo" for the purpose of the present invention is used for cells in an organism as opposed to cells growing in culture (in vitro).

The term "heterologous" with respect to the DNA sequence being operatively linked to the promoter of the invention means that said DNA sequence is not naturally linked to the regulatory sequences comprised in the recombinant DNA molecule of the invention.

In a preferred embodiment said first regulatory sequence of the invention comprises a GATA-binding site, an AP-1 binding site, an SP1 binding site, site, an NFκB binding site, a STAT binding site, a Scl/Tal-1 binding site, an Ets-1 binding site, a PEA3 consensus sequence or any combination(s) thereof. A functional analysis of the first 6.5 kbp of the transcribed region of the murine Flk-1 genes lead to the identification of a endothelial-specific positive regulatory element. This regulatory sequence is located in the region between the XhoI and BamHI restriction site in the first intron of the Flk-1 gene (cf. Fig. 4A). It is functional in both orientations since the intron enhancer was used in an antiparallel manner with respect to the Flk-1 promoter fragment in the construct referred to as 3'-In 1; see Example 2 hereinbelow. A sequence analysis of the intron lead to the identification of two potential GATA binding sites (+1927 Bp, +3514 Bp); a potential AP-1 binding site

(+2210 Bp) and two PEA3 consensus sequences (+3494 Bp, +3741 Bp); see Fig. 1. As demonstrated in Example 8, the intron sequences that were sufficient for endothelium-specific expression were contained in a 510 bp fragment (nucleotides 10094 to 10608 of SEQ ID NO: 1). Several potential binding sites for known transcription factors could be identified therein (see Figure 12), including consensus binding sites for c-ets1, PEA3 (an Ets-like transcription factor), GATA transcription factors, and Scl/Tal-1. The c-ets1 transcription factor was proposed to be involved in the early differentiation of endothelial cells from their precursors (Pardanaut Cell Adhesion and Communication 1 (1993), 151-160). In addition, c-ets1 is expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans (Wernert, Am. J. Pathol. 140 (1992), 119-127). Proteins of the Ets family can activate transcription through a PEA3 motif (Wernert, 1992). Transcription factors of the GATA family are involved in the transcription of genes that are expressed in the hematopoietic and endothelial lineages, such as *von Willebrand factor* (Jahroudi, Mol. Cell. Biol. 14 (1994), 999-1008). Unlike the hematopoietic-transcription factor GATA-1, GATA-2 is expressed in both the endothelial and hematopoietic lineages (Elefanty, Blood 90 (1997), 1435-1447). Scl/Tal-1 has recently been implicated in the regulation of *Flk-1* expression in Zebrafish (Liao, Genes Dev. 12 (1998), 621-626). The presence of two potential Scl/Tal-1 binding sites in the murine *Flk-1* intron enhancer suggests that Scl/Tal-1 might regulate *Flk-1* expression in mice. However, no direct effect of Scl/Tal-1 on *Flk-1* expression has been observed so far in mice, although Scl-null mice have vascular defects (Visvader, Genes Dev. 12 (1998), 473-479). Knock out experiments performed with the above-described regulatory sequences will easily reveal which of these elements present in, e.g., the 510 bp fragment (nucleotides 10094 to 10608 of SEQ ID NO: 1) are involved in the control of the regulatory sequence and the sequential order of these elements necessary to confer endothelium specific gene expression. Of course, the regulatory sequences obtained from such studies are also within the scope of the present invention.

Preferably, said first regulatory sequence is selected from the group consisting of

(a) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO: 1;
(b) DNA sequences comprising a nucleotide sequence of SEQ ID NO: 1 from nucleotide 8260 to nucleotide 10560, from nucleotide 8336 to nucleotide 10608 and/or from nucleotide 10094 to nucleotide 10608;
(c) DNA sequences comprising the nucleotide sequence of the human Flk-1-intron;
(d) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a), (b) or (c) under stringent conditions;
(e) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a), (b) and (c); and
(f) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (e) capable of conferring expression in endothelial cells.

In a particularly preferred embodiment of the present invention, the regulatory sequences comprise the nucleotides 8260 to 10560, 8336 to 10608 (comprising the BamHI/XhoI fragment of the first intron (+1677 bp/+3947); see Figure 4 and Examples 1 to 10), most preferably nucleotides 8560 to 10400 and still more preferably nucleotides 10094 to 10608 (comprising the SwaI/BamHI fragment (+3437 bp/3947 bp); see Example 8) of the nucleotide sequence as set forth in SEQ ID No. 1 or a fragment thereof, which still confers expression in endothelial cells, preferably at all stages of development.

In a further preferred embodiment of the invention the heterologous DNA sequence of the recombinant DNA molecules described above is operatively linked to further regulatory sequences. Expression comprises transcription of the nucleic acid molecule, preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They normally comprise promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include

transcriptional as well as translational enhancers. Preferably said further regulatory sequence is a promoter and/or a 3'-untranslated region.
Although some endothelial-specific promoters have been characterized, e.g. of the genes for von Willebrand factor (Jahroudi, Mol. Cell Biol. 14 (1994), 999-1008), Endothelin-1 (Lee, J. Biol. Chem. 265 (1990), 10446-10450), E-selectin (Collins, J. Biol. Chem. 266 (1991), 2466-2473), Tie-2 (Schlaeger, Development 121 (1995), 1089-1098), VCAM-1 (Iademarco, J. Biol. Chem. 267 (1992), 16323-16329) and endothelial NO-synthase (Zhang, J. Biol. Chem. 270 (1995), 15320-15326) these genes are neither specific for proliferating endothelium, nor necessary for endothelial cell determination. Due to the present invention these promoters can now be combined with the regulatory sequences of the invention in order to mediate endothelium specific gene expression of heterologous DNA sequences. However, other promoters can be used as well. For example, it is shown in Example 8 that the regulatory sequences of the invention conferred endothelium-specific gene expression to the heterologous herpes simplex virus-thymidine kinase (tk) promoter.

In a preferred embodiment the above mentioned promoter is a promoter of hypoxia inducible genes, genes encoding growth factors such as VEGF, PDGF or Fibroblast growth factor or their receptors or glycolytic enzymes.

In a particularly preferred embodiment said promoter comprises a DNA sequence selected from the group consisting of

(a) DNA sequences comprising the nucleotide sequence as given in SEQ ID NO:1 from nucleotide 6036 to nucleotide 6959;
(b) DNA sequences comprising the nucleotide sequence of the human Flk-1 promoter;
(c) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a) or (b) under stringent conditions;
(d) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a) and (b); and

(e) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (d).

At least one of the aforedescribed DNA sequences may be preferably of human or murine origin although other sources may be employed as well. Preferably, the heterologous DNA sequence being operatively linked to the regulatory sequences is located 5' to the regulatory sequence of the invention.

In a further preferred embodiment, the heterologous DNA sequence of the above-described recombinant DNA molecules encodes a peptide, protein, antisense RNA, sense RNA and/or ribozyme. The recombinant DNA molecule or vector of the invention can be used alone or as part of a vector to express heterologous DNA sequences, which, e.g., encode proteins other than Flk-1, in cells of the blood vessel wall, i.e., endothelial cells, for, e.g., gene therapy or diagnostics of vascular diseases such as atherosclerosis. The recombinant DNA molecule or vector containing DNA sequence encoding a protein of interest is introduced into endothelial cells which in turn produce the protein of interest. For example, sequences encoding t-PA (Pennica, Nature 301 (1982), 214), p21 cell cycle inhibitor (El-Deiry, Cell 75 (1993), 817-823), or nitric oxide synthase (Bredt, Nature 347 (1990), 768-770) may be operatively linked to the endothelial cell-specific regulatory sequences of the invention and expressed in endothelial cells. For example, thrombolytic agents can be expressed under the control of the endothelial cell-specific regulatory sequences of the invention for expression by vascular endothelial cells in blood vessels, e.g., vessels occluded by aberrant blood clots. Other heterologous proteins, e.g., proteins which inhibit smooth muscle cell proliferation, e.g., interferon-γ and atrial natriuretic polypeptide, may be specifically expressed in endothelial cells to ensure the delivery of these therapeutic peptides to an atherosclerotic lesion or an area at risk of developing an atherosclerotic lesion, e.g., an injured blood vessel.

The endothelial cell-specific regulatory sequences of the invention may also be used in gene therapy to promote angiogenesis to treat diseases such as peripheral vascular disease or coronary artery disease (Isner, Circulation 91 (1995), 2687-

2692). For example, the regulatory sequences of the invention can be operatively linked to sequences encoding cellular growth factors which promote angiogenesis, e.g., VEGF, acidic fibroblast growth factor, basic fibroblast growth factor and the like.

In a most preferred embodiment of the present invention, said protein is selected from the group consisting of Vascular Endothelial Growth Factor (VEGF), Hypoxia Inducible Factors (HIF), HIF-Related Factor (HRF), tissue plasminogen activator, p21 cell cycle inhibitor, nitric oxide synthase, interferon-γ, atrial natriuretic polypeptide and monocyte chemotactic proteins.

In another particularly preferred embodiment of the invention, said protein is a scorable marker, preferably luciferase, green fluorescent protein or lacZ. This embodiment is particularly useful for simple and rapid screening methods for compounds and substances described herein below capable of modulating the expression of genes in the endothelium. For example, endothelial cells can be cultured with VEGF in the presence and absence of the candidate compound in order to determine whether the compound affects the expression of genes which are under the control of regulatory sequences of the invention, which can be measured, e.g., by monitoring the expression of the above-mentioned marker. It is also immediately evident to those skilled in the art that other marker genes may be employed as well, encoding, for example, selectable marker which provide for the direct selection of compounds which induce or inhibit the expression of said marker.

The regulatory sequences of the invention may also be used in methods of antisense therapy. Antisense therapy may be carried out by administering to an animal or a human patient, a recombinant DNA containing the endothelial cell-specific regulatory sequences of the invention operably linked to a DNA sequence, i.e., an antisense template which is transcribed into an antisense RNA. The antisense RNA may be a short (generally at least 10, preferably at least 14 nucleotides, and up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a portion of a specific mRNA sequence. Standard methods

relating to antisense technology have been described (Melani, Cancer Res. 51 (1991), 2897-2901). Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target mRNA molecules within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA. For example, an antisense sequence complementary to a portion of or all of the Flk-1 (KDR) mRNA (Terman, Oncogene 6 (1991), 1677-1683 and Terman (1992), supra) would inhibit the expression of Flk-1, which in turn would inhibit angiogenesis. Such antisense therapy may be used to treat cancer, particularly to inhibit angiogenesis at the site of a solid tumor, as well as other pathogenic conditions which are caused by or exacerbated by angiogenesis, e.g., inflammatory diseases such as rheumatoid arthritis, and diabetic retinopathy.
The expression of other endothelial cell proteins may also be inhibited in a similar manner, for example, endothelial cell proteins such as cell cycle proteins (thereby inhibiting endothelial cell proliferation, and therefore, angiogenesis); coagulation factors such as von Willebrand factor; and endothelial cell adhesion factors, such as ICAM-1 and VCAM-1 (Bennett, J. Immunol. 152 (1994), 3530-3540).
Thus, in a further preferred embodiment of the present invention, said antisense RNA or said ribozyme are directed against a gene involved in vasculogenesis and/or angiogenesis and/or tumors of endothelial origin.

In a further embodiment, the invention relates to nucleic acid molecules of at least 15 nucleotides in length hybridizing specifically with a regulatory sequence as described above or with a complementary strand thereof. This means that they hybridize, preferably under stringent conditions, specifically with the nucleotide sequences as described above and show no or very little cross-hybridization with nucleotide sequences having no or substantially different regulatory properties. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 17, 18, 19, 20 to 25 and 25 to 35 nucleotides in length. Of course, it may also be appropriate to use nucleic acids of up to 100 and more

nucleotides in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of regulatory sequences according to the invention. Another application is the use as a hybridization probe to identify regulatory sequences hybridizing to the regulatory sequences of the invention by homology screening of genomic DNA libraries. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a regulatory sequence as described above may also be used for repression of expression of a gene comprising such regulatory sequences, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a regulatory sequence of the invention. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the presence of a nucleic acid molecule of the invention in a sample derived from an organism.

Such molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may contain, for example, thioester bonds and/or nucleotides analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell. Said nucleic acid molecules may also be transcribed by an appropriate vector containing a chimeric gene which allows for the transcription of said nucleic acid molecule in the cell. Such nucleic acid molecules may further contain ribozyme sequences which specifically cleave the (pre)-mRNA comprising the regulatory sequence of the invention. Furthermore, oligonucleotides can be designed which are complementary to a regulatory sequence of the invention (triple helix; see Lee, Nucl. Acids Res. 6 (1979), 3073; Cooney, Science 241 (1988), 456 and Dervan, Science 251 (1991), 1360), thereby preventing transcription and the production of the encoded protein.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses, bacteriophages used conventionally in genetic engineering that comprise a recombinant DNA molecule of the invention. Preferably, said vector is an expression vector and/or a targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the recombinant DNA molecule or vector of the invention into targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the recombinant DNA molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.
As is demonstrated in Example 11, the Flk-1 promoter was stimulated by HIF-2α, a basic helix-loop-helix/PAS domain transcription factor related to hypoxia-inducible factor-1. HIF-2α has previously been shown to stimulate the expression of VEGF, suggesting that HIF-2α may regulate the coordinate expression of both the VEGF receptor Flk-1 and its ligand in vivo. Thus, Flk-1 gene regulatory elements described herein can be used together with HIF-2α for the elucidation of the molecular mechanisms involved in endothelial cell specification and angiogenesis, and can be used to target expression of any transgene to the endothelium. Thus, in a preferred embodiment, the vector of the invention furthers comprises a gene capable of expressing HIF-2α.

The present invention furthermore relates to host cells transformed with a DNA molecule or vector of the invention. Said host cell may be a prokaryotic or eukaryotic cell. The vector or recombinant DNA molecule of the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. In this respect, it is also to be understood that the recombinant DNA molecule of the invention can be used for "gene targeting" and/or

"gene replacement", for restoring a mutant gene or for creating a mutant gene via homologous recombination.
The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, plant or animal cell. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae. Suitable mammalian cell lines comprise Saos-2 human osteosarcoma cells (ATCC HTB-85), HeLa human epidermoid carcinoma cells (ATCC CRL-7923), HepG2 human hepatoma cells (ATCC HB-8065), human fibroblasts (ATCC CRL-1634), U937 human histiocytic lymphoma cells (ATCC CRL-7939), RD human embryonal rhabdomyosarcoma cells (ATCC CCL-136), MCF7 human breast adenocarcinoma cells (ATCC HTB-22), JEG-3 human choriocarcinoma cells (ATCC HB36), A7r5 fetal rat aortic smooth muscle cells (ATCC CRL-1444), and NIH 3T3 mouse fibroblasts (ATCC CRL-1658) obtainable from the American Type Culture Collection. Primary-culture HUVEC may be obtained from Clonetics Corp. (San Diego, CA) and can be grown in EGM medium containing 2% fetal calf serum (Clonetics). Primary-culture human aortic and intestinal smooth muscle cells can also be obtained from Clonetics Corp. Most preferably said host cell is an endothelial cell or derived therefrom, such as BAE cells. In view of the synergistic effect of the co-expression of a recombinant DNA molecule of the invention and HIF-2α, a further embodiment of the invention concerns the above-described cells which further comprise a recombinant DNA molecule or vector containing a gene capable of expressing HIF-2α.

Moreover, the present invention relates to a pharmaceutical composition comprising at least one of the aforementioned recombinant DNA molecules or vectors of the invention, either alone or in combination, and optionally a pharmaceutically acceptable carrier or excipient. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be

effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{22} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

It is envisaged by the present invention that the various recombinant DNA molecules and vectors of the invention are administered either alone or in any combination using standard vectors and/or gene delivery systems, and optionally together with an appropriate compound, for example VEGF, and/or together with a pharmaceutically acceptable carrier or excipient. Subsequent to administration, said recombinant DNA molecules may be stably integrated into the genome of the mammal. On the other hand, viral vectors may be used which are specific for certain cells or tissues, preferably for the endothelium and persist in said cells. Suitable pharmaceutical carriers and excipients are well known in the art. The pharmaceutical compositions prepared according to the invention can be used for the prevention or treatment or delaying of different kinds of diseases, which are related to the expression or overexpression of a given gene or genes in the endothelium.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises a recombinant DNA molecule or vector of the invention in gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic

acids to a specific site in the body for gene therapy or antisense therapy may also be accomplished using a biolistic delivery system, such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).
Standard methods for transfecting cells with recombinant DNA are well known to those skilled in the art of molecular biology, see, e.g., WO 94/29469. Gene therapy and antisense therapy to prevent or decrease the development of atherosclerosis or inhibit angiogenesis may be carried out by directly administering the recombinant DNA molecule or vector of the invention to a patient or by transfecting endothelial cells with the recombinant DNA molecule or vector of the invention *ex vivo* and infusing the transfected cells into the patient. Furthermore, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., WO94/29469, WO 97/00957 or Schaper (Current Opinion in Biotechnology 7 (1996), 635-640) and references cited therein. The DNA molecules and vectors comprised in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) containing said recombinant DNA molecule into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom. The pharmaceutical compositions according to the invention can be used for the treatment of all kinds of diseases hitherto unknown as being related to the expression and/or over expression of genes in the endothelium.

The present invention also relates to diagnostic compositions or kits comprising at least one of the aforementioned recombinant DNA molecules, vectors, cells and/or nucleic acid molecules and, in the case of diagnostic compositions, optionally suitable means for detection.
Said diagnostic compositions may be used for methods of detecting and isolating regulatory sequences which are a functionally equivalent to the Flk-1 intron

regulatory sequences of the invention. The kits of the invention may further contain compounds such as further plasmids, antibiotics and the like for screening transgenic animals and/or animal cells useful for the genetic engineering of non-human animals, preferably mammals and most preferably mouse.

It is to be understood that the introduced recombinant DNA molecules and vectors of the invention express the heterologous DNA sequence after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express the heterologous DNA under the control of the regulatory sequence of the invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the recombinant DNA molecule or vector of the invention and a selectable marker, either on the same or separate vectors. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the heterologous DNA sequence under the control of the regulatory sequence of the invention, and which respond to VEGF and/or hypoxia mediated signal transduction. Such engineered cell lines are particularly useful in screening compounds capable of modulating Flk-1 gene expression.
A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt, which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072); neo, which confers resistance to the

aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1); and hygro, which confers resistance to hygromycin (Santerre, Gene 30 (1984), 147) genes. Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.). On the other hand, the person skilled in the art may also use the regulatory sequences of the invention to “knock out” an endogenous gene comprising identical or similar regulatory sequences, for example, by gene targeting, cosuppression, triple helix, antisense or ribozyme technology.

The present invention also relates to a method for the production of a transgenic animal, preferably mouse, comprising introduction of a recombinant DNA molecule or vector of the invention into a germ cell, an embryonic cell or an egg or a cell derived therefrom. The non-human animal to be used in the method of the invention may be a wildtype, i.e. healthy animal, or may have a disease or disorder, preferably a disease or disorder which is dependent on neovascularization, such as solid tumors, retinopathy, arthritis, psoriasis. Said disease or disorder may be an inborn insufficiency or natural developed or caused by genetical engineering, for instance by the expression of a DNA sequence encoding a protein involved in neuronal development and/or diseases as described above, preferably under the control of the regulatory sequence of the invention.

The invention also relates to transgenic non-human animals comprising a recombinant DNA molecule or vector of the invention or obtained by the method described above, preferably wherein said recombinant DNA molecule is stably integrated into the genome of said non-human animal, preferably such that the presence of said recombinant DNA molecule or vector leads to the transcription and/or expression of the heterologous DNA sequence by the regulatory sequence of

the invention. Further non-human animals which may be employed according to the embodiments of the invention as described above are well known to the person skilled in the art and comprise rat, hamster, dog, monkey, rabbit, pig.

With the regulatory sequences of the invention, it is now possible to study in vivo the regulation of Flk-1 expression during angiogenesis. Furthermore, since VEGF and VEGF receptor genes seem to have different functions in different stages of development, it is now possible to determine domains of said proteins which may be important for their biological activity and/or for the regulation of their activity. In addition, it is now possible to in vivo study mutations which affect different functional or regulatory aspects of VEGF or its receptor or vector of the invention.

Moreover, the present invention relates to a method for the identification of a chemical and/or biological substance capable of suppressing or activating and/or enhancing the transcription of a gene in endothelial cells comprising:

(a) contacting a cell of the invention or the transgenic non-human animal of the invention either of which is capable of expressing the heterologous DNA sequence with a plurality of compounds; and
(b) determining those compounds which suppress or activate and/or enhance the expression of said heterologous DNA sequence.

Said plurality of compounds may be comprised in, for example, samples, e.g. cell extracts from, e.g. plants, animals or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be capable of suppressing or activating and/or enhancing the transcription of a gene in endothelial cells. The plurality of compounds may be, e.g., added to the culture medium or injected into the animals.

The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which are either identical or not. If a sample containing a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating and/or enhancing the

transcription of a gene in endothelial cells, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, this can be done several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, most preferably said substances are identical.

Determining whether a compound is capable of suppressing or activating and/or enhancing the transcription of a gene in endothelial cells can be done, for example, in mice by monitoring reporter gene expression or by monitoring behavior of the transgenic non-human animals of the invention contacted with the compounds compared to that of wild-type animals or compared to a transgenic non-human animal contacted with a compound which is either known to be capable or incapable of suppressing or activating and/or enhancing the transcription of a gene in endothelial cells of said transgenic non-human animal of the invention. Furthermore, the person skilled in the art can monitor the physical behavior, or for example the movement of the above-described animals. Such methods are well known in the art. Such regulators of Flk-1 gene expression may be used in processes such as wound healing; in contrast, antagonists of expression may be used in the treatment of tumors that rely on vascularization for growth. Thus, the present invention provides methods for identifying compounds which modulate VEGF receptor (e.g., Flk-1 or Flt1) gene expression. Compounds found to downregulate expression of a VEGF receptor gene can be used in methods to inhibit angiogenesis, while compounds found to enhance Flk-1 or Flt1 expression can be used in methods to promote angiogenesis, for example, to promote wound healing (e.g., healing of broken bones, burns, diabetic ulcers, and traumatic or surgical wounds) or to treat peripheral vascular disease, atherosclerosis, cerebral vascular disease, hypoxic tissue damage (e.g., retinopathy, hypoxic damage to heart tissue), diabetic pathologies such as chronic skin lesions, or coronary vascular disease. These compounds can also be used to treat patients who have, or have had, transient

ischemic attacks, vascular graft surgery, balloon angioplasty, frostbite, gangrene, or poor circulation. Compounds identified as having the desired effect (i.e., enhancing or inhibiting Flk-1 expression) can be tested further in appropriate models of endothelial cell growth and angiogenesis which are known to those skilled in the art. Given the therapeutic value of the compounds identified in accordance with the above-described method the present invention also relates to a method for the production of a pharmaceutical composition comprising the steps of the method of the invention and formulating the compound identified in step (b) in a pharmaceutically acceptable form.
The therapeutic compounds identified using the method of the invention may be administered to a patient by any appropriate method for the particular compound, e.g., orally, intravenously, parenterally, transdermally, transmucosally, or by surgery or implantation (e.g., with the compound being in the form of a solid or semi-solid biologically compatible and resorbable matrix) at or near the site where the effect of the compound is desired. For example, a salve or transdermal patch that can be directly applied to the skin so that a sufficient quantity of the compound is absorbed to increase vascularization locally may be used. This method would apply most generally to wounds on the skin. Salves containing the compound can be applied topically to induce new blood vessel formation locally, thereby improving oxygenation of the area and hastening wound healing. Therapeutic doses are determined to be appropriate by one skilled in the art.

Furthermore, identification of transacting factors which interact with the regulatory sequences of the invention can form the basis for the development of novel therapeutics for modulating conditions associated with endothelial cell growth, such as angiogenesis, vascular disease, and wound healing. Identification of transacting factors is carried out using standard methods in the art (see, e.g., Sambrook, supra, and Ausubel, supra). To determine whether a protein binds to the regulatory sequences of the invention standard DNA footprinting and/or native gel-shift analyses can be carried out. In order to identify the transacting factor which binds to the regulatory sequence of the invention, the regulatory sequences can be used as

an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. Once the transacting factor is identified, modulation of its binding to the regulatory sequence in the Flk-1 gene can be pursued, beginning with, for example, screening for inhibitors of transacting factor binding. Enhancement of Flk-1 expression in a patient, and thus enhancement of angiogenesis, may be achieved by administration of the transacting factor, or the gene encoding it (e.g., in a vector for gene therapy). In addition, if the active form of the transacting factor is a dimer, dominant-negative mutants of the transacting factor could be made in order to inhibit its activity. Furthermore, upon identification of the transacting factor, further components in the pathway of Flk-1 signal transduction can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional drugs and methods for modulating endothelial cell growth and angiogenesis.

As discussed in the background section of the description of the present invention, the interaction of VEGF and its receptor play an important role in the onset of angiogenic disease. Transgenic non-human animals expressing VEGF and/or its receptor gene and/or mutated versions thereof under the control of the regulatory sequences of the invention can now be used for the identification of substances, which, for example, are capable of restoring the wild-type interaction of mutated VEGF and its receptor either or both of which bear mutations. Some genetic changes lead to altered protein conformational states. Genetic changes may therefore result in a decreased binding activity of VEGF. Restoring the activity of mutant VEGF protein or increasing the activity of other proteins which interact with mutant VEGF proteins is the most elegant and specific means to correct these molecular defects. In addition, some genetic changes may result in altered conformational states of the receptor. This, in turn, may functionally inactivate the tyrosine kinase activity, making it incapable of signal transduction. In order to restore the function of such mutant proteins an antibody may be used which binds to an epitope and induces a conformational change of the protein thereby restoring the wild type function. Thus, the methods of the invention are also useful to screen e.g.,

antibody, Fab, Fv or scFv expression libraries wherein the DNA sequence encoding said antibodies or derivatives thereof are under the control of the regulatory sequence of the invention. It is, of course, evident to the person skilled in the art that also other protein or peptide expression libraries using the regulatory sequences of the invention may be employed.

Further, the present invention relates to the use of the recombinant DNA molecule, vector, cell, pharmaceutical compositions, diagnostic compositions or a transgenic non-human animal of the invention for the identification of a chemical and/or biological substance capable of suppressing or activating and/or enhancing the transcription, expression and/or activity of genes and/or its expression products in endothelial cells.

In a preferred embodiment, the chemical or biological substance used in the methods and uses of the present invention is selected from the group consisting of peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, neural transmitters, peptidomimics, and PNAs (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198).

The present invention further relates to a method of inhibiting a vascular disease in a subject, comprising contacting an artery of said subject with the recombinant DNA molecule or vector of the invention, wherein said protein reduces or prevents the development of the vascular disease, preferably said protein reduces proliferation of smooth muscle cells.

In a further embodiment the present invention relates to the use of a recombinant DNA molecule, vector, nucleic acid molecule of the invention and/or substance identified by a method of the invention for the preparation of a composition for directing and/or preventing expression of genes specifically in endothelial cells and/or for the preparation of a pharmaceutical composition for treating, preventing and/or delaying a vascular disease and/or a tumorous disease in a subject. The

upregulation and activation of the Flk-1 receptor in peri-tumoral endothelial cells is believed to be involved in the neovascularization of various human or experimental tumors (Plate, 1994; Ferrara, Curr. Opin. Nephrol. Hypertens. 5 (1996), 35-44). This hypothesis is supported by experiments in which the inhibition of Flk-1-mediated signal transduction strongly inhibits tumor angiogenesis and tumor growth (Millauer, Nature 367 (1994), 576-579; Millauer, Cancer Res. 56 (1996), 1615-1620). Thus, by using compounds of the present invention described above capable of inhibiting Flk-1 gene expression, it is possible to ameliorate tumorous diseases which depend on the expression of the FLK-1 gene.

In a further embodiment, the present invention relates to the use of a recombinant DNA molecule, vector and/or the nucleic acid molecule of the invention for the preparation of a pharmaceutical composition for inducing a vascular disease in a non-human animal or in a transgenic non-human animal described above.

In a preferred embodiment of the methods and uses of the invention, the vascular disease is atherosclerosis and/or a neuronal disorder. Further possible methods and uses in accordance with the present invention will be evident to the person skilled in the art and are described in, for example, WO 95/13387, WO 94/11499 and WO 97/00957.

The recombinant DNA molecules, vectors, nucleic acid molecules, compounds, uses and methods of the invention can be used for the treatment of all kinds of disorders and diseases hitherto unknown as being related to or dependent on the modulation of genes specifically expressed in the endothelium. The recombinant DNA molecules, vectors, nucleic acid molecules, compounds, methods and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the methods and uses described herein. Thus, the present invention provides for the use of a regulatory sequence as defined above for enhancing and/or directing gene expression in endothelial cells in any kind of organism.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The figures show:

Figure 1: Nucleotide sequence of the murine Flk-1 gene. The ATG codon is at position +299. The three exons are indicated in bold. Motifs for transcription factors are underlined. VRE: vascular response element,

Figure 2: Map of reporter gene construct pGL2-B. Arrows symbolize functional elements. Luc: luciferase gene, AMP: ampicillin resistance gene, f1ori: replication origin for bacteriophage f1.

Figure 3: Map of reporter gene construct pGLacZ. Arrows symbolize functional elements. LacZ: β-galactosidase gene, AMP: ampicillin resistance gene, f1ori: replication origin or bacteriophage f1.

Figure 4: Partial structure and functional analysis of the mouse Flk-1 locus. A) Restriction enzyme map of the region encompassing the first three

exons (represented by hatched boxes). Subfragments containing parts of intron 1 or intron 2 are indicated. Abbreviations for restriction enzymes are: B, BamHI, Xh, XhoI, SI, SalI. B and C, luciferase reporter gene assays of various constructs following transient transfection of bovine aortic endothelial cells. B) Transfection assay of the intron fragments in combination with the Flk-1 promoter region of bp –640 to bp +299. The values were coordinated with 5'-In1 fragments with respect to the activity of the construct. C) The intron fragments were tested in combination with a 4.4 kbp Flk-1 promoter fragment spanning the region from -4.1 kbp to + 299 bp of the Flk-1 gene. NIH 3T3 cells were used as a reference for non-endothelial cells. RLU, relative light units

Figure 5: Analysis of the intron in transgenic mice. The embryo (10.5 days) was stained overnight with X-Gal. The reporter gene was under the control of the intron enhancer (3'-In1, cf. Fig. 4A) and of the Flk-1 promoter fragment ranging from nucleotides -4100 to +299. A) Top lateral view. B) Top dorso-cranial view.

Figure 6: Histological evaluation of a transgenic embryo. The embryo shown in Fig. 5 was embedded in paraffin. The cuts were stained with neutral red. A) Pseudo transversal cut through the head region. B) Magnification of a section from A. C) Pseudo transversal cut of a caudal region. 1: 4th ventricle of cerebrum, 2: acoustic vesicle/otocyte, 3: V. cardinalis anterior. 4: third ventricle of cerebrum. 5: endbrain vesicle. 6: optic vesicle. 7: ganglion trigeminale (V). 8: chorda dorsalis.

Figure 7: Functional analysis of the first two introns of the Flk-1 gene in vivo. The depicted embryo (10.5 days) carries the ß-galactosidase gene under the control of the Flk-1 promoter (-4.1 kbp to +299 bp) and the

first 6.2 kbp of the transcribed region (cf. Fig. 4A). The staining was carried out as described in Fig. 5.

Figure 8: In vivo characterization of the intron enhancer in combination with the strongest promoter fragment. All three embryos carry the ß-galactosidase gene under the control of the Flk-1 promoter fragment of bp -640 to bp +299 and the intron enhancer. The staining was carried out as described in Fig. 5.

Figure 9: Detailed analysis of the left-hand embryo from Fig. 8A) Left lateral view. B) Sectional magnification of A. C) Right lateral view.

Figure 10: Histological evaluation of the embryo depicted in Fig. 9. The embryo was embedded in paraffin and was cut into 10 μm slices. The cuts were stained with neutral red. A) Pseudo transversal cut through the head region. B) Magnification from a similar cut level as in A. C) Pseudo transversal cut from a more caudally located section. D) Pseudo transversal cut from thoracal section. 1: 4th ventricle cerebrum, 2: 3rd ventricle cerebrum, 3: endbrain vesicle, 4: A. carotis interna, 5: ganglion trigeminale (V), 6: V. cardinalis anterior, 7: neural tube, 8: esophagus, 9: V. cardinalis posterior, 10: aorta dorsalis, 11: endocardium of the heart atrium, 12: vessels of the myocardium.

Figure 11: Reporter gene analysis of Flk-1 gene regulatory elements in transgenic mouse embryos. The lacZ reporter gene was fused to regulatory elements derived from the mouse Flk-1 gene and tested for β-galactosidase expression in transgenic mouse embryos. A) 10.5 day transgenic mouse embryo expressing *lac*Z under the control of a 939 bp promoter fragment in combination with a 2.3 kbp XhoI/BamHI fragment of the first intron spanning the region from +1677 bp to +3947 bp of the *Flk-1* gene. This embryo was derived from a foster

mother. Most if not all developing vascular structures show β-galactosidase expression, for example the endocardium of the heart, the dorsal aorta, intersomitic vessels or vessels of the developing brain. B) 11.5 day embryo of a transgenic mouse line that was established with the same construct. C) An 11.5 day *Flk-1/lacZ* knock-in embryo in which the *lac*Z gene is expressed from the endogenous *Flk-1* locus shows a highly similar staining. However, note the absence of β-galactosidase expression in small blood vessels of the yolk sac. D-F) Paraffin sections of the β-galactosidase stained embryo from (B) demonstrate β-galactosidase expression in the paired dorsal aortae (D), a venous vessel connected with the heart (E), and capillaries invading the neural tube (F). G) β-galactosidase expression in a transgenic embryo containing the *tk* promoter in combination with the 2.3 kbp XhoI/BamHI fragment of the *Flk-1* first intron. H) β-galactosidase expression in a transgenic embryo containing a construct with *Flk-1* promoter sequences (-640 bp/+299 bp) in combination with the 510 bp SwaI/BamHI fragment of the first intron spanning the region from +3437 bp to +3947 bp of the *Flk-1* gene. Bar D) –F) 100 μM.

Figure 12: Nucleotide sequence of the *Flk-1* intron enhancer and putative transcription factor binding sites. Sequences matching known transcription factor binding sites are underlined. This sequence is deposit in the GeneBank database (accession number AF061804).

Figure 13: Analysis of transgene expression during early development and in newborn mice. The transgenic mouse line 2603 expresses *lac*Z under the control of the *Flk-1* promoter (-640 bp/+299 bp) in combination with the 2.3 kbp *Flk-1* intronenhancer A) Frontal view on a whole mount β-galactosidase stained 7.8-day embryo. The arrow indicates transgene expression in the extraembryonic mesoderm. B) and C)

Paraffin sections from the embryo shown in A demonstrate transgene expression in endothelial cells of the allantois (B) and the yolk sac (C). D-H, LacZ staining of spleen (D), kidney (E), lung (F), liver (G) and thymus (H) from a postnatal day 5 transgenic mouse. EM, extraembryonic mesoderm. Bars, 25 μM (C), 100 μM (B,D,E,F,G,H).

Figure 14: The 5'-UTR is required for expression of the *Flk-1* gene in the yolk sac vasculature. Transgenic mouse embryos that contain a *Flk-1* promoter and 5'UTR (-640 bp/+299 bp) / enhancer (+1677 bp/+3947 bp) reporter gene construct show a complete vascular expression in the yolk sac vasculature (A and B). In contrast, the yolk sac of *Flk-1/lacZ* knock-in embryos which lack part of the 5'UTR show expression only in large collecting vessels that connect with the embryo, but not in the smaller vessels (C). Bar, 500 μM.

Figure 15: HIF-2α stimulates *Flk-1* gene expression. A293 cells were co-transfected with a reporter gene construct containing *Flk-1* promoter sequences from bp -640 to bp +299 and with expression vectors encoding the murine HIF-1α and HIF-2α cDNAs, respectively. Relative promoter activities were determined as described in Materials and Methods. The promoter activity of the control transfection was arbitrarily set to 1.

The examples illustrate the invention.

Example 1: Cloning and construction of Flk-1 intron/reporter gene vectors

DNA clones containing the 5' region of the mouse Flk-1 gene were isolated from a library prepared from 129/SvJ mice in λ Dash II vector (Stratagene) (Rönicke, supra) or in λ FIX II or obtained from the P1 Library (Genome Systems, St. Louis). A 21 kb region of the mouse Flk-1 gene, contained in the DNA insertions of two λ phages 6

and 16, including approximately 15 kb of 5' flanking sequences, exons 1, 2 and 3, and introns 1 and 2 was characterized by restriction enzyme mapping and Southern blot analysis. Lower DNA fragments of the phage clones were cloned into pBluescript vector DNA (Stratagene) and used for further characterization. Sequencing was performed using an automatic Sequencer (373A, Applied Biosystems). The nucleotide sequence of the *Flk-1* intron enhancer is deposited in the Genbank database (accession number AF061804). The search for potential transcription factor binding sites was performed with the MatInspector software (Quandt, (1995) Nucl. Acids Res. 23, 4878-4884).

The DNA sequence (SEQ ID NO: 1) of a 12.8 kb region spanning from about position -6,660 kb (relative to the transcriptional start site) to approximately position +6,135 kb (located in the third exon) was determined (Fig. 1). Figure 4A shows a schematic representation of the first 6.5 kbp of the transcribed region of the murine Flk-1 gene. Exons I, II and III are emphasized as hatched boxes. The first intron having a length of 3.6 kbp is subdivided into two regions (5'-In1 and 3'-In1). The region In-2 contains the entire second intron, the second exon, the 3' end of the first intron and part of the third exon. This subdivision into various intron fragments was maintained in the following analyses. The reporter gene constructs used were derived from pGL2 basic vector (Promega) that contains a promoterless luciferase gene. Luciferase reporter gene constructs were generated for transfection of cells in vitro. For use in transgenic mice in vivo, plasmids were used in which the luciferase reporter gene was replaced by a lacZ reporter gene.

In order to generate (luciferase) reporter gene constructs, Flk-1 promoter fragments were amplified by PCR and cloned into pGL2 (Promega) vector DNA 5' to the luciferase gene as described by Rönicke, supra; see also Figure 2. In short, the upstream primers used were -1900: 5'-GGG GTA CCG AAT TCT AAA TGG GGC GAT TAC C-3' (SEQ ID NO 2); -640: 5'-GTG GTA CCC AAA CAC TCA ACA CCA CTG-3'(SEQ ID NO: 3); -624, 5'-TCG GTA CCG ACC CAG CCA GGA AGT TC-3' (SEQ ID NO: 4); the downstream primer was +299, 5'-TTG CTA AGC TTC CTG CAC CTC GCG CTG GG-3' (SEQ ID NO: 5). To generate the construct ranging from -4100 to +299, a HindIII-EcoRI fragment of recombinant lambda phage 6 from P1

Library (Genome Systems, St. Louis) was inserted into the plasmid ranging from -1900 to +299. Vectors that contained Flk-1 intron sequences in addition to promoter sequences were generated as follows: specific intron sequences were amplified by PCR from cloned Flk-1 genomic DNA and inserted downstream of the reporter gene. Primers used for amplification were 5'-In1down: 5'-AGG GAT CCA CTC TTT AGT AGT AAG GCG-3' (nucleotides 7036-7057 of SEQ ID NO: 1, SEQ ID NO: 6); 5'-In1up: 5'-ACC TCG AGA CTT GGA TGG CAC-3' (nucleotides 8324-8342 of SEQ ID NO: 1, SEQ ID NO: 7); 3'-In1down: 5'-GGG CTA TAA TTG GTG CCA TCC-3' (nucleotides 8312-8332 of SEQ ID NO: 1, SEQ ID NO: 8); 3'-In1up: 5'-GGA TGG AGA AAA TCG CCA GGC-3' (nucleotides 10637-10658 of SEQ ID NO: 1, SEQ ID NO: 9); IN2A: 5'-GTG TGC ATT GTT TAT GGA AGG G-3' (nucleotides 10571-10593 of SEQ ID NO: 1, SEQ ID NO: 10); IN2B: 5'-CAT AGA CAT AAA CAG TGG AGG C-3' (nucleotides 12849-12871 which is part of the cDNA sequence published by Millauer (1993), supra, SEQ ID NO: 11). For the subsequent experiments the vector indicated in Fig. 3 was used. It represents a modification of the pGL2 basic vector in which the corresponding Flk-1 promoter fragments were inserted into the KpnI and HindIII restriction sites of the polylinker (Fig. 2). Also the luciferase reporter gene was replaced by the ß-galactosidase gene (Schlaeger, Proc. Natl. Acad. Sci. USA 94 (1997), 3058-3063). For an analysis of the intron intron fragments were cloned into the BamHI and SalI restriction sites indicated. DNA manipulations, PCR amplification and DNA sequencing were performed according to conventional methods known in the art as described, for example in Sambrook, supra and PCR Technology, Griffin and Griffin, eds., RC Press London (1994).

Example 2: Functional analysis of the intron of the Flk-1 gene in vitro

Figure 4 shows the result of transient transfections in BAECs. The corresponding intron fragments were combined with a Flk-1 promoter fragments which comprised nucleotides -640 to +299. The promoter activity was standardized with respect to the promoter activity of the construct containing the 5'-In1 fragment.

Tissue culture and transient transfections were performed as follows:

All cells were cultured in DMEM+ supplemented with 10% FCS (Sigma) and as described in Schaeger, 1997. bEnd5 cells were generated by transformation with the Polyoma middle-T oncogene as described earlier (Montesano, Cell 62 (1990), 435-445). Bovine aortic endothelial cells (BAECs) were prepared as described (Schwartz, In Vitro 14 (1978), 966-980). NIH 3T3, C2C12 and L cells were obtained from ATCC. Transient transfections were performed using the $CaPO_4$-precipitation method according to Chen and Okayama (Mol. Cell Biol. 7 (1987), 2745-2752), optionally with modifications as described (Rönicke, 1996). The transfection efficiency was monitored by co-transfection of a β-galactosidase reporter vector.
Each construct was transfected at least six times in three independent experiments. Cells were grown to 70% confluence in 6-cm dishes prior to transfection. Cells were washed 16 hrs after addition of CaPO-precipitate and incubated for further 48 hrs. In each experiment, 6 μg luciferase and 1 μg pCMV5 (Rönicke, supra) lacZ reporter gene constructs were used. Cells were lysed in 1 x reporter-lysis-buffer (Promega) for 15 min on a test tube-rotator. After centrifugation, the supernatant was transferred to a fresh tube and stored at -80 °C or taken for luciferase-and lacZ-assay immediately. Reporter-gene assays for β-galactosidase activity were performed according to Eustice (Biotechniques 11 (1991), 739-740). Chlorophenol red-β-D-galactopyranoside (CPRG) was used as a substrate and the conversion was measured at 575 nm in an ELISA-reader (Biometra). Extracts were diluted to obtain OD_{575}nm values between 0.2 and 0.8. These values were used to standardize for transfection efficiency after subtracting a background value, as determined from a cell extract of a transfection without lacZ-reporter plasmid but with a luciferase-reporter plasmid. Luciferase-reporter gene assays were performed with the same extracts as described by the manufacturer (Promega). Luciferase activity was measured with a luminometer (LB96P, Berthold) and calculated as per cent of the activity of the pGL2-promoter plasmid (Promega).

Construct	5'-In1	3'-In1	In2
BAEC	100+/-0%	128+/-34%	136+/-52%
3T3	100+/-0%	55+/-15%	74+/-33%

Table I: Functional analysis of the intron of the Flk-1 gene. The upper line indicates the corresponding intron fragment which was analyzed in combination with the Flk-1 promoter (-640 bp/+299 bp).

Figure 4C shows the results of another transfection assay of the intron fragments. It was carried out as described above, with the exception that a Flk-1 promoter fragments was used that comprised the region between nucleotides -4100 and +299. Also, a fragment was analyzed that contained the entire first intron, the second exon, the second intron and part of the third exon shown in Figure 4A.

Construct	5'-In1	3'-In1	In2	In1+2
BAEC	100+/-0%	206+/-81%	119+/-51%	154+/-68%
3T3	100+/-0%	71+/-32%	85+/-27%	35+/-12%

Table II.: Functional analysis of introns of the Flk-1 gene. The upper line indicates the corresponding intron fragment which was analyzed in combination with the Flk-1 promoter (-4100 Bp/+299 Bp).

An analysis of this experiment revealed that the construct with the 3' region of the first intron in BAECs had an activity that was twice that of that containing the 5' region of the first intron. Also, it showed 85% higher activity than the construct with the second intron (p=0.0153). The 4.5 kbp longer construct In1+2 that also

contained the 3' region of the first intron, too, revealed an activity that was markedly higher in BAECs than in 3T3 cells.

A functional analysis of the first 6.5 kbp of the transcribed region of the murine Flk-1 genes lead to the identification of an endothelial-specific positive regulatory element. This regulatory sequence is located in the region between the XhoI and BamHI restriction site in the first intron of the Flk-1 gene (cf. Fig. 4A). It is functional in both orientations since the intron is used in an antiparallel manner with respect to the Flk-1 promoter fragment in the construct referred to as 3'-In 1. In construct In1+2, however, the original orientation was maintained. A sequence analysis of the intron enhancer lead to the identification of two potential GATA binding sites at position +1927 bp and +3514 bp; (Evans, Proc. Natl. Acad. Sci. USA 85 (1988), 5976-5980; Orkin, Blood 80 (1992), 575-581), a potential AP-1 binding site at position +2210 bp; (Lee, Cell 49 (1987), 741-752) and two PEA3 consensus sequences at position +3494 bp and +3741 bp; (Martin, Proc. Natl. Acad. Sci. USA 85 (1988), 5839-5843).

Example 3: Functional characterization of the Flk-1 promoter in vivo.

So far, analyses of the murine Flk-1 promoter have been restricted to in vitro systems (Rönicke, supra; Patterson, supra). The investigation of the promoter activity in vitro is an important tool in promoter characterizing since it is useful to assay a large number of promoter constructs for their activity in a short time. However, this situation is always an artificial one since not all factors that are relevant in vivo can also be reconstituted in vitro. While an in vitro investigation of a promoter yields important information on the mechanisms of gene regulation it is only the in vivo characterization that can yield the final proof for the relevance of the elements identified. An excellent test system for promoter analysis in vivo are transgenic mice. In this model the corresponding promoter fragment was cloned before a reporter gene, isolated together with this reporter gene and injected into fertilized mouse oocytes. In many cases, successful integration of the promoter reporter construct into the mouse genome lead to transgenic mice which contain the

construct in every cell. This test system, in addition to the analysis of the promoter activity during embryonic development and in the adult animal, allows a tissue-specific characterization of the promoter activity.
For the investigation of the Flk-1 promoter in transgenic mice the bacterial ß-galactosidase reporter gene was chosen since the gene product is easily detectable by color reaction and remains at the location of production due to its limited solubility. In this manner it is possible to identify cells in which the corresponding Flk-1 promoter fragment since only there an expression of ß-galactosidase took place.
When producing transgenic mice it was taken care that no regions originating from the vector were injected along with the promoter. First, Flk-1 promoter fragments comprising the regions between nucleotides -640 and +299, -1900 and +299 as well as -4100 and +299 were investigated. The constructs were based on plasmid pGL-2B described in Figure 2 with the exception that the luciferase reporter gene was replaced by the ß-galactosidase gene. All injection fragments used in the examples were obtained by restriction digestion with the enzymes KpnI and SalI. Transgenic mice were generated as described by (Hogan, Manipulating the Mouse Embryo (1994), Cold Spring Harbor Laboratory Press, New York). Fertilized oocytes were isolated from superovulated C57BL/6 x C3H/He F1 mice, microinjected and reimplanted into pseudopregnant females of the same hybrid-mouse strain. Mice were sacrifized at day 10.5 or 11.5 of gestation, and embryos were analyzed by whole mount LacZ staining for transgene expression. The embryos to be examined were isolated on day 10 after reimplantation of the injected oocytes. Analysis of the transgenic embryos revealed that although promoter activity could be detected, none of the constructs was capable of conferring reproducible expression of the reporter gene in the endothelium.

Example 4: Functional characterization of the Flk-1 intron in vivo

After analysis of the Flk-1 promoter region from -4.1 kbp to +299 Bp the intron which was identified in vitro was then examined for its function in vivo. For this purpose, a construct similar to that shown in Figure 3 was used which contained an Flk-1

promoter fragment ranging from nucleotide -4100 to base pair +299 and the intron enhancer (3'-In1, cf. Fig. 4A). The staining and fixation of the embryos was performed as follows: The mid-day of the plug observation was counted as E0.5. The embryos were dissected out in ice-cold PBS and fixed in ice-cold 2 % (w/v) paraformaledehyd, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 M Pipes buffer, pH 6.9 for 15 to 120 minutes. The embryos were rinsed with PBS three times for 5 minutes each. The LacZ expression was detected by incubating the embryos at 30 °C overnight in 0.1 % X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide 5 mM, 1 to 2 mM magnesium chloride, 0.002 to 0.02 % NP-40, 0.01 % or 0.25 mM sodium deoxycholate, PBS, pH 7.0. After the staining, embryos were rinsed in PBS and postfixed at 4 °C overnight in 2 % paraformaldehyde, 0.1 % glutaraldehyde, PBS, pH 7.0. For whole-mount photography, the postfixed embryos were rinsed in PBS and equilibrated for 3 minutes each in (optionally 30 %) 50 % glycerol and then in 70 % glycerol. Figure 5 shows an embryo (embryonic day 10.5) which was isolated after injection of the fragment. In Figure 5A a color reaction in vessels of the developing brain can be clearly discerned. Also superficial vessels in the body's middle (dorsal) and a staining in the liver bud can be observed. Figure 5B shows the dorsal, caudal region of the same embryo. It proves that the vessels in both halves of the head were stained.

For an exact localization of the stained cells the embryo was embedded in paraffin, cut into slices of 10 μm and counterstained with neutral red. Cryostat sectioning and lacZ staining of organs from postnatal mice was performed as described (Schlaeger, 1997).

The results of the histological analysis are shown in Figure 6. It shows pseudo transversal cuts of the embryo. In section 6A a staining of the inner lining of the V. cardinalis anterior (3) and of other superficial vessels can be seen. Figure 6B represents a strong magnification of a section of 6A with the staining of endothelial cells within the V. cardinalis anterior. Figure 6C shows a more caudally located region. Again, the staining of the V. cardinalis anterior and of superficial vessels with

wide lumen and thin walls as well as of vascular structures in the neural tube is clearly visible. Also, a staining of the chorda dorsalis (9) can be observed. However, in none of the cases a staining of arterial vessels could be observed.
The subsequent injection of the same fragment lead to a total of eight further transgenic embryos which displayed an identical expression pattern albeit in two cases of weaker nature. Thus, the intron enhancer exhibited in vivo an effect that was even more marked than in vitro. In combination with a promoter fragment which on its own had a very variable expression pattern it ensures a reproducible expression pattern with clear endothelium specificity, however, covering a substantial part of the endogenous Flk-1 expression pattern.

Example 5: Functional analysis of the introns I and II of the murine Flk-1 gene in vivo

Since the intron enhancer in combination with an Flk-1 promoter fragment displayed an endothelium-specific function in transgenic mice covering a substantial part of the endogenous expression pattern, the further search for in vivo relevant, gene regulatory elements was extended to other intron regions. For this purpose, the construct containing the promoter region between nucleotides -4100 to +299 and the first 6.5 kbp of the transcribed region (In1+2; cf. Fig. 4) was used. Figure 7 shows an embryo on embryonic day 10.5 which was obtained after injection of this fragment. Again, a staining of the vessels in the developing brain as well as superficial vessels the of the liver bud was visible. The following injections yielded four further transgenic embryos which displayed the same pattern. A combination of the promoter region used with only the 5' end of the first intron (5'-In1; cf. Fig. 4), however, yielded no endothelium-specific expression pattern.

Example 6: Combination of the intron enhancer with the Flk-1 promoter fragment that was the most potent in vitro

To investigate whether the repressing elements of the murine Flk-1 promoter between nucleotides -4100 and -640 are functional also in combination with the intron enhancer, a shorter construct without these inhibitory regions was used for further analysis. It contain the intron enhancer (3'-In1) and the 5' region from base pair -640 to nucleotide +299. This 5' region displayed the highest activity in vitro. Figure 8 shows three transgenic embryos (embryonic day 10.5) which were obtained after injection of the fragment. All three display a more marked staining in vascular structures than the embryos analyzed so far. While the embryo on the right hand shows a weak staining, the left-hand embryo yields a very strong expression in virtually all vessels. The embryo in the middle holds a medium position as regards the completeness of its expression pattern, i.e., it lacks expression in the heart although it resembles strongly the embryo on the left hand as regards the staining of the other structures. In Figure 9A the left-hand embryo from Fig. 8 is shown in more detail. The strong staining of the heart in the region of the atrium and ventricle is particularly clearly visible. Furthermore, the vessels of the developing brain, the vessels between the somites, the aorta dorsalis as well as the fine capillary plexus on the body's surface are stained. Figure 9B shows a sectional magnification of 9A. Here, the staining of the vessels in the head region as well as the expression in the superficial capillary plexus is visible. In Figure 9C the same embryo is shown from the other side. In addition to the structures described in Figure 9A also a staining of the chorda dorsalis can be observed.

The embryo shown in Figure 9 was embedded in paraffin and cut to slices. The cuts dyed with neutral red are shown in Fig. 10. Figure 10A shows a pseudo transversal cut through the head region. Particularly prominent is the branching of the A. carotis interna (4) in addition to the staining of other vascular structures. Figure 10B represents a magnification of a similar cut; here, too, the branching of the A. carotis interna is particularly striking. Figure 10C shows a more caudal cut which in terms of its position roughly corresponds to the cut shown in Figure 6A. Here, however, in

addition to the staining of the V. cardinalis anterior (6) an expression in the branching of the A. carotis interna (4) and other vascular structures is visible. Figure 10D represents an even more caudally located region. A staining in the venous endothelium (V. cardinalis posterior, 9) and in the arterial structures (aorta dorsalis, 10) can be observed. Furthermore, the endocardium of the atrium as well as the vessels in the trabeculae of the heart ventricles display an expression.

A total of seven transgenic embryos was analyzed after injection of this fragment (-640 bp/+299 bp/3'-In1). Safe for one which showed no staining, all embryos displayed an expression of the ß-galactosidase in endothelial structures. The staining was regularly more marked than in combination with the negative regulatory elements between nucleotides -4100 and -640. Thus, the in vitro identified regions displayed a function in vivo. The deletion of these negative regulatory elements yielded a construct that lead to a reproducible expression in venous and arterial endothelium.

Example 7: Endothelium-specific expression mediated by Flk-1 regulatory sequences in vivo

When the Flk-1 promoter fragment with the strongest in vitro activity (-640 bp/+299 bp; Rönicke, supra) was tested in combination with the 2.3 kbp XhoI/BamHI fragment of the first intron that showed endothelium-specific activity in vitro (3'-In1; +1677 bp/+3947 bp; see Example 6), a reproducible vascular lacZ expression in transgenic E10.5 mouse embryos derived from foster mothers was observed (Table III). In these embryos, the lacZ reporter gene was expressed in developing vascular structures, such as capillaries in the head region, intersomitic vessels, the dorsal aorta, and in the heart anlage (Fig. 11A). Sectioning of these embryos confirmed that the β-galactosidase protein was confined to vascular endothelium. This in vivo analysis demonstrated that the intron sequences in combination with the Flk-1 promoter confer an endothelium-specific expression pattern that closely resembles the expression pattern of the endogenous Flk-1 gene (Millauer, 1993). Moreover, the intron fragment could also direct endothelial cell-specific lacZ expression when used

in an inverted orientation in the reporter construct (Construct -640 bp/+299 bp//+3947 bp/+1677 bp; see Table III).

Table III. Summary of the in vivo activity of different flk-1 constructs

Construct	TG	ES	ET	NO
-4100/+299	11	0	3	8
-1900/+299	31	1	10	20
-640/+299	3	0	1	2
-640/+299 // 3´Intron +1677/+3947	7	6	0	1
-640/+299 // 3´Intron +3947/+1677	4	3	1	0
-640/+299// 3´Intron +3437/+3947	7	5	0	2
-640/+254 // 3`Intron +1677/+3947	12	8	1	3
tk // 3`Intron +1677/+3947	15	3	0	12
-5500/+299 // Intron I+II	3	2	0	1

Embryos transgenic for the constructs given above were generated, and LacZ staining and genotyping was performed at E10.5 or E11.5 as described in Example 4. Constructs are defined by the position of the promoter or intron fragments in bp relative to the transcription initiation site of the endogenous Flk-1 gene. TG, number of transgenic embryos; ES, number of embryos showing endothelial-specific staining; ET, number of embryos showing ectopic staining; NO, number of embryos showing no staining at all.

Transgenic mouse lines were generated with this reporter gene construct (-640 bp/+299 bp//+1677bp/+3947bp) containing the *Flk-1* regulatory sequences. One of these lines (2603) showed a complete vascular expression of the reporter gene at

E11.5 and was analyzed further (Fig. 11B). Sectioning of β-galactosidase stained E11.5 transgenic embryos revealed that reporter gene expression was confined to the endothelium of blood vessels, e.g. in the endothelium of the dorsal aorta (Fig. 11D), in venous vessels (Fig. 11E) and in the perineural vascular plexus and sprouting capillaries invading the neural tube (Fig. 11F). To determine whether transgene expression in this mouse line reproduced the complete expression pattern of the endogenous *Flk-1* gene, the lacZ staining pattern of these embryos was compared to heterozygous *Flk-1* mutant mouse embryos which express the *lac*Z gene from the endogenous *Flk-1* locus (Shalaby, Nature 376 (1995), 62-66). In these knock-in mice, the *lac*Z gene was inserted into the endogenous *Flk-1* locus via homologous recombination and is therefore expected to reproduce the expression pattern of the endogenous *Flk-1* gene (Shalaby, 1995). The lacZ staining pattern of transgenic embryos and the knock-in embryos at E11.5 was indistinguishable (Fig. 11B,C). It is concluded from these data that the -640 bp/+299 bp promoter region of the *Flk-1* gene and the 2.3kbp XhoI/BamHI fragment of the first intron contain regulatory elements that are sufficient for endothelial-specific gene expression in developing mouse embryos.

Example 8: The first intron of the Flk-1 gene contains an autonomous endothelium-specific enhancer

To assess the role of the 2.3 kbp XhoI/BamHI fragment of the first *Flk-1* intron in endothelium-specific gene expression, it was further investigated whether the intron sequences can confer endothelium-specific expression to the heterologous herpes simplex virus-thymidine kinase (*tk*) promoter. This promoter has no intrinsic endothelial cell specificity (Schlaeger, 1997). A *lac*Z reporter gene construct was generated that contained the *tk* promoter, in combination with the 2.3 kbp BamHI/XhoI fragment of the first intron (+1677 bp/+3947 bp). The *tk* promoter sequences were amplified from the plasmid ptkSDKlacZ (Schlaeger, 1997) using oligonucleotides tk5' (5'-CCGGTACCCAAACCCCGCCCAGCGTCTTG-3'; SEQ ID

NO: 16) and tk3' (5'-CCGACAAGCTTGGTCGCTCGGTGTTCGAGG-3'; SEQ ID NO: 17). The PCR product was digested with KpnI and HindIII.

From the β-galactosidase reporter construct described in Example 2 (Fig. 2), Flk-1 promoter sequences were exised and removed by KpnI and HindIII digestion. The *tk* promoter was then subcloned in the KpnI and HindIII restriction sites of the vector. Transgenic mouse embryos generated with this construct showed vascular reporter gene expression (Fig. 11G). The β-galactosidase staining observed in these embryos was weaker than in embryos expressing *lac*Z under the control of the -640 bp/+299 bp *Flk-1* promoter in combination with the intron fragment (Fig. 11A,B). Also, the frequency of transgenic mouse embryos expressing this transgene was significantly reduced, when compared with constructs driven by the -640 bp/+299 bp *Flk-1* promoter in combination with the intron fragment (Table III). This indicates that the *tk* promoter lacks positive acting elements which are present within the *Flk-1* promoter. However, these results show that the *Flk-1* intron fragment alone, in contrast to the *Flk-1* promoter, can reproducibly target reporter gene expression to the endothelium. Taken together, the results of both the in vitro an in vivo experiments in this study demonstrate that sequences located in the first intron of the mouse *Flk-1* gene act as an autonomous endothelium-specific enhancer.

In order to further characterize the minimal intron sequences that are required for endothelium-specific expression, we analyzed whether shorter intron fragments were also active in combination with the 939 bp promoter region of the *Flk-1* gene (-640 bp/+299 bp). By this deletion analysis, the intron enhancer was localized to a 510 bp SwaI/BamHI fragment (+3437 bp/+3947 bp) which is located immediately upstream of the second exon. This fragment was sufficient to stimulate endothelium-specific *lac*Z expression in transgenic mouse embryos (Fig. 11H, Table III). The DNA sequence of this fragment (Fig. 12) contains potential binding sites for the GATA and Ets transcription factors, and for Scl/Tal-1, all of which have been implicated to play a role in angiogenesis (reviewed by Risau, Nature 386 (1997), 671-674). Whether these consensus sequences represent functional transcription factor binding sites remains to be determined.

Example 9: Flk-1 regulatory sequences target endothelium-specific transgene expression throughout development

To test whether the regulatory sequences of the *Flk-1* promoter and enhancer identified can reproduce the endogenous *Flk-1* expression pattern throughout development, the *lac*Z expression pattern of the transgenic mouse line 2603 (Fig. 11B) was further analyzed at various stages of embryonic development and at postnatal days 5 (P5) and 120 (P120). In this mouse line, the transcription of *lacZ* is driven in combination by the -640 bp/+299 bp *Flk-1* promoter and the 2.3 kbp BamHI/XhoI intron enhancer fragment. The earliest stage during which transgene expression was detectable by whole mount LacZ staining was in E7.8 embryos (Fig. 13A). This is the earliest stage that was examined. The analysis of sections of these embryos confirmed that the transgene was expressed in angioblasts of the allantois and the yolk sac (Fig. 13B,C). Moreover, transgene expression was restricted to the vascular endothelium at all stages of embryonic development examined. To determine if the transgene expression persists after birth, we performed lacZ staining of cryostat sections from several different organs of P5 and P120 transgenic mice. Strong LacZ staining was detected in vessels of the spleen, kidney, thymus, liver and lung from P5 animals (Fig. 13D-H). However, *lac*Z expression was downregulated in most vascular beds of P120 animals, as is the case for the endogenous *Flk-1* (Millauer, 1993; Kremer, Cancer Res. 57 (1997), 3852-3859). Taken together, these results support the conclusion that the identified *Flk-1* regulatory sequences (the 939 bp promoter in combination with the intron enhancer) are sufficient to reproduce most, if not all, properties of the endogenous *Flk-1* expression.

Example 10: The 5'-UTR of the Flk-1 gene is required for expression of Flk-1 in the yolk sac vasculature

In *Flk-1*/*lac*Z knock-in embryos, the *lac*Z gene is under control of all endogenous regulatory elements except for the regions from bp +137 to bp +299 in the 5'-UTR

and approximately the first 600 bp of the first intron (Shalaby, 1995). It has been shown in accordance with the present invention that the intron sequences deleted in the knock-in construct created by Shalaby (1995) are not required to generate the strong and complete endothelial-specific reporter gene expression which is mediated by the *Flk-1* regulatory sequences described in this study (Fig. 11B and Table III). However, since the complete *Flk-1* 5'-UTR is present in the reporter gene construct which directs the most complete vascular-specific *lac*Z expression (-640 bp/+299 bp//+1677 bp/+3947 bp; Fig. 11B and Table III), it allows to study the consequences of a partial 5'-UTR deletion on *Flk-1* expression in vivo: Genomic DNA was prepared from unstained embryos or yolk sacs. Genotyping was performed by PCR analysis using the primer pairs -258fw/LacRev or LacZP1/LacZP2. Primers for PCR analysis were: -258fw: 5'-ATGGTACCCAGGTTGCTGGGGGCAG-3' (SEQ ID NO: 12); LacRev: 5'-TGGTGCCGGAAACCAGGCAAA-3' (SEQ ID NO: 13); LacZP1: 5'-ATCCTCTGCATGGTCAGGTC-3' (SEQ ID NO: 14); LacZP2: 5'-CGTGGCCTGATTCATTCC-3' (SEQ ID NO: 15). The complete vascular staining of the *Flk-1/lac*Z knock-in embryos at E11.5 indicates that the 5'-UTR is not essential for vascular expression in the embryo proper. However, the yolk sac staining pattern of *Flk-1/lac*Z knock-in embryos and of transgenic mice from this study that harbored constructs containing the complete 5'-UTR were markedly different (Fig. 14A-C). The uniform vascular *lac*Z expression in the transgenic yolk sacs from this study (Fig. 14A, B) was absent in small vessels of the yolk sacs of the knock-in embryos (Fig. 14C), in which only large yolk sac vessels were stained. In addition, it was found that replacement of the entire *Flk-1* promoter including the 5'-UTR by the *tk* promoter in the present transgenic construct (Table III) leads to a similar *lac*Z expression pattern in the yolk sacs as that described in the yolk sacs of the *Flk-1/lac*Z knock-in embryos. Thus, the 5'-UTR might be involved in specifying *Flk-1* expression in a subset of endothelial cells.

Example 11: The role of HIF-2α in Flk-1 regulation

The *Flk-1* promoter (-640 bp/+299 bp) confers endothelium-specific expression to the firefly luciferase reporter gene in transfected bovine aortic endothelial cells (Rönicke, 1996) and provides for a strong reporter gene transcription in vivo; see Examples 6 to 10. This suggests that transcription factors that are specifically expressed in endothelial cells activate the *Flk-1* promoter in a cell-type specific manner.

The basic helix-loop-helix PAS-domain transcription factor, HIF-2α (also known as HLF, HRF or EPAS1), is expressed in endothelial cells during mouse embryonic development (Ema, Proc. Natl. Acad. Sci. USA 94, 4273-4278, 1997; Flamme, Mech. Dev. 63 (1997), 51-60; Tian, Genes Dev. 11 (1997), 72-82) and is thus a candidate to regulate *Flk-1* expression. HIF-2α has previously been shown to stimulate both the expression of *VEGF* (Ema, 1997) and *Tie2* (Tian, 1997). To determine if HIF-2α might be involved in the regulation of *Flk-1* gene expression, A293 cells were co-transfected with a luciferase reporter gene construct containing *Flk-1* promoter sequences (-640 bp to +299 bp) and an eukaryotic expression vector that contained the mouse HIF-2α cDNA. Mouse HIF-2α and HIF-1α cDNAs were obtained from a mouse brain capillary endothelial cell cDNA library (Schnürch, Development 119 (1993), 957-968) with a 300 bp BamHI/NcoI fragment spanning the 5'UTR of HIF-1α. Positive phages were rescreened, and inserts were amplified by PCR using oligonucleotides HIF Start: (5'-GGGAATTCACCATG AGTTCTGAACGTCGAAAAG-3'; SEQ ID NO: 18) and HIF Flag Stop: (5'-AAGCGGCCGCTCATTTATCGTCATCGTCCTTGTAATCGTTAACTTGATCCAAAG CTCTG-3'; SEQ ID NO: 19). The PCR product was digested with Eco RI and NotI and subcloned in the EcoRI and NotI restriction sites of pcDNA3 expression vector. The murine HIF-2α cDNA was obtained as described (Flamme, 1997). The phage insert was amplified by PCR using oligonucleotides HRF START (5'-GGGAATTCACCACAATGACAGCTGACAAGGAG-3'; SEQ ID NO: 20) HRF rev (5'-AAGCGGCCGCTCATTTATCGTCATCGTCCTTGTAATCGTTGGTGGCCTGGTCCA GAGCTCTGAG-3'; SEQ ID NO: 21) and PCR product was digested with EcoRI/NotI

and cloned into the EcoRI and NotI sites of pcDNA3. The sequence encoding the FLAG epitope was included in the reverse oligonucleotide primer. HIF-2α and HIF-1α expression plasmids were constructed by inserting the FLAG-tagged cDNAs into the EcoRI and NotI sites of pcDNA3 (Invitrogen). For co-transfection assays, A293 cells were split 1:2 into 35 mm dishes and transfected 18 hours later with 4 μg of DNA (2 μg of *Flk-1* promoter-driven luciferase plasmid, 1 μg of CMV promoter-driven β-
galactosidase expression plasmid, and 1 μg of the HIF-2α or HIF-1α expression plasmids, or pBluescript SKII and pcDNA3 as a control) using a transfection kit (MBS, Stratagene). After 20 hours, reporter gene activity measurements were performed using the Dual Light Kit (Tropix, Bedford, MA). The luciferase activity of each extract was normalized to the respective β-galactosidase activity. Endogenous background levels of both enzyme activities were measured using extracts from mock-transfected cells and were substracted. The normalized luciferase activity of the control transfection was arbitrarily set to 1. Each value represents the average of at least six experiments.

In comparison to cells transfected with the luciferase reporter construct alone, co-transfection of the HIF-2α construct increased reporter gene activity approximately 15-fold (Fig. 15). In contrast, HIF-1α , a close relative of HIF-2 which stimulates the hypoxia-induced transcription of the *VEGF* gene, failed to stimulate the reporter construct (Fig. 15). These results suggest that HIF-2α regulates the expression of the *Flk-1* gene.

Summary

The mouse Flk-1 receptor is crucial for the differentiation of the hemangioblastic lineage and during embryonic vascular development (Risau, Annu. Rev. Cell Dev. Biol. 11 (1995), 73-91; Shalaby, 1995; Risau, 1997). Moreover, Flk-1 plays a central role in the regulation of neovascularization in a wide variety of tumors (Plate, Brain Pathol. 4 (1994) , 207-218; Ferrara, 1996). To elucidate the basis of its endothelial expression, regulatory sequences of the murine *Flk-1* gene have been isolated and

characterized that confer endothelium-specific reporter gene expression in transgenic mouse embryos. Transgene expression driven by these sequences was strong, specific, and highly reproducible. Most importantly, it has been demonstrated that the isolated sequences were active in early stage vascular development and may thus represent a clue towards the identification of the molecular mechanisms involved in hemangioblast differentiation and vasculogenesis. Moreover, transgene expression persists until shortly after birth and is downregulated in adult animals, as it was described for the endogenous *Flk-1* gene (Millauer, 1993; Kremer, 1997).
Endothelium-specific expression in almost all transgenic mouse embryos tested was mediated by a 939 bp fragment of the promoter region in combination with a fragment of the first intron. 5'-flanking fragments up to -5.5 kbp alone were not sufficient to confer a reproducible endothelium-specific transgene expression. Reproducible endothelium-specific expression was therefore dependent on sequences from the first intron. These sequences also activated the heterologous *tk* promoter specifically in endothelial cells in vivo, and were active in an orientation independent manner. Thus, they fulfill the criteria for an autonomous tissue specific enhancer.
As demonstrated in Example 8, the intron sequences that were sufficient for endothelium-specific expression were contained in a 510 bp fragment. Several potential binding sites for known transcription factors could be identified therein (see Figure 12), including consensus binding sites for c-ets1, PEA3 (an Ets-like transcription factor), GATA transcription factors, and Scl/Tal-1. The c-ets1 transcription factor was proposed to be involved in the early differentiation of endothelial cells from their precursors (Pardanaut, Cell Adhesion and Communication 1 (1993), 151-160). In addition, c-ets1 is expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans (Wernert, Am. J. Pathol. 140 (1992), 119-127). Proteins of the Ets family can activate transcription through a PEA3 motif (Wernert, 1992). Transcription factors of the GATA family are involved in the transcription of genes that are expressed in the hematopoietic and endothelial lineages, such as *von Willebrand factor* (Jahroudi, Mol. Cell. Biol. 14 (1994), 999-1008). Unlike the hematopoietic-transcription factor

GATA-1, GATA-2 is expressed in both the endothelial and hematopoietic lineages (Elefanty, Blood 90 (1997), 1435-1447). Scl/Tal-1 has recently been implicated in the regulation of *Flk-1* expression in Zebrafish (Liao, Genes Dev. 12 (1998), 621-626). The presence of two potential Scl/Tal-1 binding sites in the murine *Flk-1* intron enhancer suggests that Scl/Tal-1 might regulate *Flk-1* expression in mice. However, no direct effect of Scl/Tal-1 on *Flk-1* expression has been observed so far in mice, although Scl-null mice have vascular defects (Visvader, Genes Dev. 12 (1998), 473-479).

Recently, analyses of the regulatory elements of other endothelium-specific genes such as *von Willebrand factor* (Aird, Proc. Natl. Acad Sci .USA. 92 (1995), 4567-4571), *c-ets-1* (Jorcyk, Cell. Mol. Biol (Noisy-le-grand) 43 (1997), 211-225) or the endothelial receptors, *Tie1* (Korhonen, Blood 86 (1995), 1828-1835) and *Tie2* (Schlaeger, Development. 121 (1995), 1089-1098; Schlaeger, 1997) have been reported. The most uniform expression pattern reported was conferred by regulatory elements of the *Tie2* gene. However, in contrast to *Flk-1,* expression of *Tie2* and of reporter genes driven by *Tie2* regulatory sequences is not downregulated in adult animals. Such as in the *Flk-1* gene, the first introns of the *Tie2* gene and of the *Ets-1* gene are involved in endothelium-specific expression. Similar to the *Flk-1* intron enhancer, the first intron of the Tie2 gene also contains an autonomous endothelial specific enhancer. A major difference between the structural organisation of the regulatory elements of the *Flk-1* gene and the *Tie2* gene is, however, that the *Tie2* promoter by itself is active in certain embryonic blood vessels (Schlaeger, 1995). At least during the developmental stages analyzed (i.e. E10.5 and E11.5) an autonomous function of the *Flk-1* promoter was not observed. The intronic 303 bp Tie2 core enhancer also contains potential binding sites for transcription factors of the Ets and GATA families (Schlaeger, 1997), and c-ets1 or PEA3 binding sites are present in the promoters of *Tie1*, *Tie2* and *Flt-1* (Korhonen, 1995; Schlaeger, 1995; Wakiya, J. Biol. Chem. 271 (1996), 30823-30828).

Analysis of *Flk-1/lacZ* knock-in mouse embryos that express the *lacZ* gene from the endogenous *Flk-1* locus has previously shown that the *lacZ* reporter gene is expressed ubiquitously in the developing intra-embryonic vasculature and the yolk

sac of E7.5 embryos (Shalaby, 1995). However, in accordance with the present invention it was found that a fragment of the 5' UTR that is deleted in the knock-in construct is required for reporter gene expression in the yolk sac vasculature during later stages of embryonic development. Based upon transient transfection analyses in bovine aortic endothelial cells, the *Flk-1* 5'-UTR has been shown to contain a positive acting, endothelial cell-specific element between nucleotides +136 and +299 (Rönicke, 1996). The complete vascular staining of the *Flk-1/lacZ* knock-in embryo proper at E11.5 demonstrates that the 5'-UTR is not essential for intraembryonic vascular expression at this developmental stage.

The involvement of HIF-2α in the regulation of *Flk-1* expression further emphasizes the role of basic helix-loop-helix/PAS-domain transcription factors in the regulation of components of the VEGF signal transduction system and of vascular development. The upregulation of VEGF in response to hypoxia is generally thought to be mediated by HIF-1. Moreover, mouse embryos lacking functional genes for HIF-1α or ARNT show defects in vascular development, perhaps due to reduced VEGF levels (Maltepe, Nature 386 (1997), 403-407; Iyer, Genes Dev. 12 (1998), 149-162). This observation indicates that the physiological relevance of these transcription factors is not restricted to adaptation to hypoxia, but extends to the regulation of normal vascular development. HIF-2α is expressed in various tissues, including the developing endothelium of several organs, for example in the brain (Flamme, 1997). It seems therefore likely that HIF-2α is involved in the regulation of *Flk-1* expression in blood vessels that co-express *HIF-2*α and *Flk-1*. Interestingly, *HIF-2*α is also expressed in tissues that express the Flk-1 receptor ligand, VEGF, and has been shown to stimulate *VEGF* expression (Ema, 1997). Taken together, these observations support the hypothesis that HIF-2α is both an intrinsic and extrinsic regulator of blood vessel growth and function (Flamme, 1997), by stimulating both receptor and ligand expression. The expression of VEGF and Flk-1 shows a remarkable coordinate temporal pattern both in development and in tumors. For example, VEGF and Flk-1 are expressed transiently in the developing mouse brain, and are largely down-regulated in the adult, but reactivated in brain tumors (Plate, 1994). In hemangioblastomas of the brain, which are highly vascularized tumors,

both the *VEGF* and *Flk-1* expression are highly up-regulated, and this correlates with the up-regulation of *HIF-2α* expression in the stromal cells of this tumor type. Whether HIF-2α contributes to the remarkably coordinated expression of *VEGF* and *Flk-1* in other tumor types, remains to be established, since for example in glioblastomas - another cerebral tumor - up-regulation of *VEGF* is due to hypoxia, and HIF-2α is inducible by hypoxia. Unlike the expression of *VEGF* and *Flt-1*, *Flk-1* expression is not directly stimulated by hypoxia (Gerber, J. Biol. Chem. 272 (1997), 23659-23; Kremer, 1997). Thus, the primary function of HIF-2 in the regulation of *Flk-1* expression does not appear to be related to the hypoxia response.

Among the endothelial RTK identified thus far, Flk-1 is the only receptor whose function is required for the determination of the endothelial lineage. Therefore, the *Flk-1* gene represents the ideal candidate for studying the transcriptional regulatory mechanisms that are active during the emergence of the endothelial lineage. The observation that the isolated regulatory elements of the *Flk-1* gene are active in early stage vascular development are of great importance for this objective. Knowledge of the *Flk-1* gene regulatory sequences is also of great potential relevance in the therapy of certain diseases. The Flk-1 receptor has been demonstrated to be a key regulator of angiogenesis in various diseases, including cancer (Plate, 1994). Therefore, the study of the regulatory elements involved in the upregulation of *Flk-1* expression in the tumor endothelium appears to be particularly relevant for studying the mechanisms of tumor angiogenesis. Further studies will unravel whether the same regulatory elements of the *Flk-1* gene that confer endothelium-specific expression in mouse embryos are also active in the tumor vasculature. *Flk-1* gene regulatory elements active in the tumor vasculature may provide information about the signaling pathways that can be targeted for anti-angiogenic tumor therapy. Finally, the *Flk-1* gene regulatory elements will be useful for targeting expression of genes to the vasculature. An attractive possibility is the expression of suicide genes (Ozaki, Hum. Gene Ther. 7 (1996), 1483-1490) under the control of these elements. The use of the *Flk-1* gene regulatory elements in combination with, e.g., the Cre/loxP system may provide a powerful tool for specifically inactivating genes in the developing vasculature or in tumor endothelium.

The present invention is not to be limited in scope by its specific embodiments described which are intended as single illustrations of individual aspects of the invention and any DNA molecules, or vectors which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described therein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Said modifications intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Regulatory Sequences Capable Of Conferring Expression Of A Heterologous DNA Sequence In Endothelial Cells In Vivo And Uses Thereof

(iii) NUMBER OF SEQUENCES: 21

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12845 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTAGAATAT AGAAGATAAG TTTGCGTACA ATTCAGTCCT TTGAAGACCT GATAAGCTTT 60

AAGAAGGAAG ATGGGTTACA CATTGGGAAA TGGTTGCAAT CTGCACATGG CAGAGGCAAG 120

AGATGCAAAT CACATTTCTT ACATACTCCA TACAAATCTT ACAAGACTGT TTTTCTTTCT 180

CATTTAAAAT AAGAAGACCT GCCAGTCTTC CCCTTATTAC TAATTACAGT CACTCTGTAT 240

CTTTGTTGAC ATTGGATAGT TTTACATACT TCAACAGGCT GGTGTCATTA AAGTTGTGGT 300

GGGTGGGCAC CAGAGACACG TGATTCAGAG TGGGAGGAGA TGCAGGAGAA ACGAGGCACA 360

GCAGAAGCAG AAGCGAGGAA AAACACTCTC AACGTTACTA ACACATCGAG AGGTTCCGCA 420
CACTAGCAAT ACGGGCTGAA TCTGACCTAA TCTCTGCTGT TGAAAATTTT GCCTAGCCGC 480
ACACTAGCAA TACGGGCTGA ATCTGACCTA ATCTCTGCTG TTGAAAATTT TGCCTAGCCT 540
GTCACACAAG TGCTGAGCAT ACAGAAAAAG GAGAGTAATT CTCTGGTTCT TTGACTAACC 600
AAATAGTCTA TATCAAATTG CCTAAGATAA TGTATACATT TAGTACATGA CTGGTTATAC 660
CTATTCTATA TGACTATTAT TTAAATGTGA ATTTACAAGT GAGCATATGA AGTCCATTTT 720
ACATGGCTAG TACATATAAC TTTTAAAAAG TTGGACATAG TTATATTTTT CCATTTATTT 780
ATTTACTTTA TATCCTGATC ACAGACCCCC CCCTCCTCTG GATTAACTCT CTCCACTGCT 840
TCTTACCCCT CCCCATCTCT CCTTCACCTC TGAGAAGGGG GGATACCTCC TGTCTTATCT 900
GGTTTCAGTG GGAGAAGGAT GTATCCTAAC ACATATAATT TTTAATATCC TGAGTTTTTC 960
TTTCATACAC CTTACTTATT CTATTCATTT TTCAGGAAGG CATGTTTAAT GTTTTTTTTT 1020
TAATTTTATG TGTACGAGTG TTTTGCCTAC ACAGTCATAG TGCATCGCAT ACATTTTTGC 1080
TGCCCGTAGA GATCAGAAGG GAGCATTGGG TTCCCTAGGA CTGGAGGCAT GAACCACCTT 1140
GTGGGTGCAG AGAACTGAGC CTGGGTCATC TCAAAGCATC AGGTTCTTCT TGAGTCATCT 1200
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TGCTAGCCCC ACCCCATACT ATCTTTATAA TATCTGTTTA ATTAAGACAT TCATAATGAA 1440
TTTTATTAAC ATTCATCGTT ATCCCCTTTA CCAATTTTAC TATGTATTAA TTGCCACCCC 1500
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CCTAAATGGA CTTGCTTGTT ATTCATTTAC AGTGTGGCCC CTTGACTTTC ATCGGCACTC 2820

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TTATCCCTAC ACAGGCCCTG GAGCCAGTTC CCAGCACCCA CACGGTGGCT CACAACCATC 2940

TGTAACTCCA GTTCTAGGAG ACCCGACTCC CTCTTCTGTC TGAAAACACC AGGCACGCGT 3000

GCGGTCTACA TACAAACATG AAAGCAAAAT ACACACATTA CATAAATAAA TCTTAAAAAA 3060

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CACTCATCAC ACTGTAAAGT AGAATTGAAC TCTGACAGAA CAAGCGAAGT GAGTCTGACT 3780

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ACTTGGGCAT GGTGAGAAGG AAACAACGCA GGAGGGCTAG CCAAGTCTGA GAGTCGTGAG 3900

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TCCGTTTGGG CTGGCGCGCC TTGGGGATCC TCCTATCTCT CCTTCCCCAG TGCTGGGATA 4500

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CTAAATGGGG CGATTACCAC GGGCCTGCAG GTTCTGGTTT GTATTAGAGG AGACACTGTC 4860

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ACCGGTTTCC TCTTTGTTCC TACACTGGCG CCTCTGGCAA AAAACTCTCC CTTCTCTTCC 5820
CCCCAAGCAT ATCTTGGCTG AAAGGTCAGC TCTGAAAAGG GGCCTGGCCA AAGTTACTGT 5880
AGGGGACCGT GGTCATGGAA CTGGGTAAAC AAAAGCACTC TAGCAGCCAC TGGAAAAGGA 5940
CCGGGGGCTC TTCTCTGTGC ATTTGCCCTG GAACCCTGAC CACCGCCAGC TCCCTGCATC 6000
TCCTTGCTAT GGGTTTTCTG GACCGACCCA GCCAGGAAGT TCACAACCGA AATGTCTTCT 6060
AGGGCTAATC AGGTAACTTC GGACGATTTA AAGTTGCCAG ATGGACGAGA AAACAGTAGA 6120
GGCGTTGGCA ACCTGGATAA GCGCCTATCT TCTAATTAAA ACATTCAGAC GGGGCGGGGG 6180
ATGCGGTGGC CAAAGCACCA TAAAACAAAA CTTCCAAGTA CTGACCAACT CACTGCAAGT 6240
TTGTGCCCCG AGTACATCTA GGTTCAGGGG TTCTTGTCTT CATGCTCCCA ACTGCGGGCG 6300
GATTTTTGGT CCCTTGGGAC TTTCAGTGCA GCGGCGAAGA GAGTTCTGCA CTTGCAGGCT 6360
CCTAATGAGG GCGCAGTGGG CCTCGTGTTT CTGGTGATGC TTCCCAGGTT GCTGGGGGCA 6420
GCAAGTGTCT CAGAGCCCAT TACTGGCTAC ATTTTACTTC CACCAGAAAC CGAGCTGCGT 6480
CCAGATTTGC TCTCAGATGC GACTTGCCGC CCGGCACAGT TCCGGGGTAG TGGGGGAGTG 6540
GGCGTGGGAA ACCGGGAAAC CCAAACCTGG TATCCAGTGG GGGGCGTGGC CGGACGCAGG 6600
GAGTCCCCAC CCCTCCCGGT AATGACCCCG CCCCCATTCG CTAGTGTGTA GCCGGCGCTC 6660
TCTTTCTGCC CTGAGTCCTC AGGACCCCAA GAGAGTAAGC TGTGTTTCCT TAGATCGCGC 6720
GGACCGCTAC CCGGCAGGAC TGAAAGCCCA GACTGTGTCC CGCAGCCGGG ATAACCTGGC 6780
TGACCCGATT CCGCGGACAC CGCTGCAGCC GCGGCTGGAG CCAGGGCGCC GGTGCCCCGC 6840
GCTCTCCCCG GTCTTGCGCT GCGGGGGCGC ATACCGCCTC TGTGACTTCT TTGCGGGCCA 6900
GGGACGGAGA AGGAGTCTGT GCCTGAGAAC TGGGCTCTGT GCCCAGCGCG AGGTGCAGGA 6960

TGGAGAGCAA GGCGCTGCTA GCTGTCGCTC TGTGGTTCTG CGTGGAGACC CGAGCCGCCT 7020

CTGTGGGTAA GAAGCCCACT CTTTAGTAGT AAGGCGGAGA AGTAGGGTGC GGGCGGAGAG 7080

TGGGAATAGA AGAGGACCTA ACTCGTAGAG CTCTAGAGAC CCTCCTCCCT TGGGTGTTCT 7140

TTCACTTACC AATGGGGAAA CTGAGGTTCA AAGACTCTTC CGAAATGACT CAGCCAGGAT 7200

TCTACTCTCC CCCGGGCATC GGTTGGAGCG TGTCCTGCGG AGCCGTCACA GCCCCTGGCG 7260

CTAGGTAGGC AGGAGTGGAA AGGCGGCCTG AGCCGGGGCA GGAGATGCTC CCACTGGCAG 7320

GAACAGGCGG TCAAACGCTG GGAAGCCAGC TCAAGCCAAG CGGCCCGGCT GGCATCAATC 7380

ACTCGGTGCT GTTGCCCACC GCCCTAGTGG GGGGCAGGGA ATCCGCCTCT GGCTCCGCTC 7440

CCCTTTAGCT CCAGCGTGTA AGCGCACGGA CTATGTGAGG GTAGGTCTCT TCATAGAGCA 7500

ACACTTTCCT CCCTCAACTT TCTTTGATGC AGAATGCTAT TTTTGCTGGT AGGAGGAAGA 7560

CGCGGCTTTC TCTTCTGTGA CAGCTTCTCC AGGTGTATTA AACTAAATAA CTCTCCACTT 7620

ACCGACTCCA AAGCGCTGGT CCTGGGGTAA ACTCTGAAAG TCTCAGAAAC TCTTGAGCTT 7680

GGCACCTAGT TATAGGTCAC TTTTCTTGTT TTAAAATGCC CTCTGCTTCA AGGTTAGGCC 7740

CACACTCGCT CTTGGGCTTT TGTGCAATAA TTTCCCTTCC CTTCCCTTCC CTTCCCTTCC 7800

CTTCCCTTCC CTTCCCTTCC CTTCCCTTCC CTTCCCTTTC CCTCTTCCTT TTCCTCCTCC 7860

TCTTCCTCCT CTATTTCTCT GTCATTTCCT TTTTGAAGCC ACAGTTTGCA GATTTCCAAT 7920

CTCCACCCAT TGGAGAATGG AGAATCAGGA AAAAAGAAGT CAATTCTGCA GAAACATTCC 7980

TTGCGCCCTA AGAGAATCGC ATGGCTTAAA AGCATTGGCA CTGACATACG GCGCCAAGAT 8040

CGCCTGTCTA GAGCTATTGA GTTTTCCTCA TAATGACTTG GTTCATCAGG CTAGCTCCAC 8100

CACGAGTGCC CTCTTGTTCC TGAGAAGGCC GCACTCTCCC CCTTTCTGGG AAGAGAAAGA 8160

CAGCCTGGAA CATGTGCTTG CCCTGGGTTC CATAGAGAAG CAAGTTGCTT TAAAGCCCAG 8220

AGAATTCCTA GTGTAGCAGC TTAACAGCGT CCCGTTCTCT GAATAAGATG GAGGTTGCCC 8280

TTTTGGAGTG TGTGACTTGC TTAATTGGAT TGGGCTATAA TTGGTGCCAT CCAAGTCTCG 8340

AGACAGAGCC GCTGTTGTTT TTCCTTCTGG TCTTTGAGCG GGAAGGATAA CAGTGCACAA 8400

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AAGATGAGCA TAGCCAACGT GCCTGCACTC TAAGAGAATC TATGGCTGTA TGTTATTACA 8700

GAGACAGTTG AGAAGCTCTT AGTGGCTCTG GCGTGTAGAT CAGCGGTAGA GCGCTGAGGC 8760

TCTGCGCTCG CTTCCTGGCA CTGAAGAATA AAGGCCATTT ACTGTGGTGG TGCAGTGGGC 8820

GCAGTTTGTG ACGAGTTACT ACTACATTTT CCTCACACAT CTGCCTGACT AATGAGTTCA 8880

TCAGATGAGC GTATCCAGTG ATTGTTTGCA GGTTAATGGT TCTCAGTCAT GTTTAGAATC 8940

TACTTATCAA ACAAATTGTT TTCTCATTTC CTGCTTCTTC TCAAACAAAG TAAGATTCCA 9000

TTATTGAAAG GCTTGTTTAA GAGCATTTTA ACTGCTTGCC TATGTTAGGG ACAGTGACTT 9060

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CTTTAACAAT GCCAACTAGC TTCTGAGATT CATTAATGTC ATTTAATTAC CAATGTTTTA 9300

AAAATATGTC ATTAATTACT AAATCTATAG TTGTAACAGC AACACATGTA CATCTTATTA 9360

AGTTGGGTAT ATTCAGGGTG GCATAGCTGT AGACTATTGC ACATCTGTGT TGGTGAGCCA 9420

GTGGAGAACT GCCTCCTGGC TGTTCTCAGA AGGCCACAGT GTCACGGCAT TGGCTATTTG 9480

CCTTGGCTCT TTGCTAATAC TTTATTGACA TGGCCTCATC TTCGTTCACG TTCACTTATT 9540

TGCCCAACAA CGTCAATGCC AGCTGAGGCC TTAGGAGTCA TCTGTTCTTA GTCAGTGCGA 9600

ATTAGAAAGC CTGGATGCCT GCCTGCTATT AATTAGTTAT TCTTCTCTTC TGAGACAGAG 9660

TCTCACTGTG TGGCCCAGGC TAGTCTCAAA CTTGCGGTCC ATTTGTCTCA CTCATCAGAA 9720

TGCTGGGCTT CCAGGTGTGT GCACCACACT AGGTAGCTCG CGTTTTAAGC TAAGAGCTGG 9780

AAGATCCTGA TGTCCTTTAC CATGGTGGGC ATGTTACAGG TTAGTTGACT GAAAACTAGT 9840

TATCTCGCTG TGTAATGACC TGCAGTGGTA TGTATCTCTC AAGATGCTTT TTTGCATTTC 9900

AATCAGTTAG GTAACAAGTT CTTAAGTCTC CAGCTTGGTA TTGGCATGAG CTCAGAGCTT 9960

TGATTAATGA GTTGGGACCC CCTAGCTATT GCTCATTAGA CTTACACTAT TTTTAGTTTT 10020

GCTCTGAGTT TATGAATATG CATGTATGCA TGAACTTGGG AGATATTTTT CTTCCCCAAT 10080

TCCTTTTCCT CCATTTAAAT GTGCTGTCTT TAGAAGCCAC TGCCTCAGCT TCTGCAGCTC 10140

AGATACCAAA GGAAGTCTGG TACACAGCAT GATAAAAGAC AATGGGACGG GGTCACAGTG 10200

GCTCCCGTCC CTTTCAGGGG TATGGAGACG AGCTGTAGAG AGATGTCTCC AGGGAGTTTT 10260

CATTAATCAG CAATTTAGTC AGATCTGTGC ATCCTATGCT TTACAAGAAA TGTCAGTGGG 10320

CCTGAGATCA TCAGATGGAG GTTCATCGGG TTTCAATGTC CCGTATCCTT TTGTAAGACC 10380

TTGAAGTTGG CAACGCAGGA AAACAGGAAC TCCACCCTGG TGCCGTGAAT TGCAGAGCTG 10440

TTGTGTTGGT TTGTGACCAT CTGCCCATTC TTCCTGTTAT GACAGAGCTT GTGAACTTTA 10500

ACTGGGACTG GGGCAAAGTC AATCCCACCT TTATACAATG AATTGCTGAA GAGGCCTTTT 10560

AAAACTTGGA GTGTGCATTG TTTATGGAAG GGCTTTCCTA TTGGATCCAA CTCTTTTCTA 10620

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ACATACTGAC AATTTTGGCA AATACAACCC TTCAGATTAC TTGCAGGTAA GGATTCCTTT 10740

TTGAGCCAGC TTTCCTATGT GAAAGGACTC ATTGTTTACT GAGGTCACAA CAATTTCCAC 10800

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CTATTTAATA ATGAAACAAT GGAAAAAGTC TTTTCAAACC TTTGTACTCT TTTGCTGAGC 10920

CGTTTTCAAC ATGCACAAAC ATATTACACA AATATAACAT ACACAGGAAC ACACATGAAT 10980

GCATGGGATG ATGTGCCTAA AACTAGCATG TAATTGATAT TCACAATTAT TGATAAATTA 11040

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GAATGGCTTC TGGACATTTT TTTTTTTAGC TGTTTTCTAC AAGTGAAATT CTGCCTGTAT 11160

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CATGAAAGAC CTGTTTGCAG GGCAGTGTGT TTTGCTGGGG ACAGTAATGA GCCTAGATCG 11460

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CCTCATATTT GAAGAATACT AAGAGGTCCC CCGAATAACT CAGGGCTAGT GTTGATCATT 11580

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TTTGGGGGAA AGCAAAACCA GAAAGCGATG ATAGCAGGAC CTGTTTATTT TCATTAAGTC 11700

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CCTCTGGAGA CTGCTGGGAA CATTTCTTAA GTTCTGAAAA AACCCTGGAG TGATTGGGCA 11820

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CTCATAGCCT CTCTTAATCA TAGCCCTGTG GCATGGAGTG TACCATTGAT ATCTTCCTGG 12660

AATACTTTTT CAGGGGACAG CGGGACCTGG ACTGGCTTTG GCCCAATGCT CAGCGTGATT 12720

CTGAGGAAAG GGTATTGGTG ACTGAATGCG GCGGTGGTGA CAGTATCTTC TGCAAAACAC 12780

TCACCATTCC CAGGGTGGTT GGAAATGATA CTGGAGCCTA CAAGTGCTCG TACCGGGACG 12840

TCGAC 12845

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGGGTACCGA ATTCTAAATG GGCGATTAC C 31

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTGGTACCCA AACACTCAAC ACCACTG 27

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCGGTACCGA CCCAGCCAGG AAGTTC 26

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTGCTAAGCT TCCTGCACCT CGCGCTGGG 29

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGGATCCAC TCTTTAGTAG TAAGGCG 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACCTCGAGAC TTGGATGGCA C 21

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGGCTATAAT TGGTGCCATC C 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGATGGAGAA AATCGCCAGG C 21

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTGTGCATTG TTTATGGAAG GG 22

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CATAGACATA AACAGTGGAG GC 22

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ATGGTACCCA GGTTGCTGGG GGCAG 25

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGGTGCCGGA AACCAGGCAA A 21

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

....(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATCCTCTGCA TGGTCAGGTC 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

....(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGTGGCCTGA TTCATTCC 18

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGGAATTCAC CATGAGTTCT GAACGTCGAA AAG 33

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AAGCGGCCGC TCATTTATCG TCATCGTCCT TGTAATCGTT AACTTGATCC AAAGCTCTG 59

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGGAATTCAC CACAATGACA GCTGACAAGG AG 32

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGCGGCCGC TCATTTATCG TCATCGTCCT TGTAATCGTT GGTGGCCTGG TCCAGAGCTC 60

TGAG 64

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCGGTACCCA AACCCCGCCC AGCGTCTTG 29

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CCGACAAGCT TGGTCGCTCG GTGTTCGAGG 30

Claims

1. A recombinant DNA molecule comprising:
   (a) at least one first regulatory sequence of an intron of the Vascular Endothelial Growth factor (VEGF) receptor-2 (Flk-1) gene or of a gene homologous to the Flk-1 gene being capable of conferring expression in endothelial cells in vivo; and
   (b) operatively linked thereto a heterologous DNA sequence.

2. The recombinant DNA molecule of claim 1, wherein said first regulatory sequence comprises a GATA-binding site, an AP-1 binding site, an SP1 binding site, an NFκB binding site, a STAT binding site, a Scl/Tal-1 binding site, an Ets-1 binding site, a PEA3 consensus sequence or any combination(s) thereof.

3. The recombinant DNA molecule of claim 1 or 2, wherein said first regulatory sequence is selected from the group consisting of
   (a) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO: 1;
   (b) DNA sequences comprising a nucleotide sequence of SEQ ID NO: 1 from nucleotide 8260 to nucleotide 10560, from nucleotide 8336 to nucleotide 10608 and/or from nucleotide 10094 to nucleotide 10608;
   (c) DNA sequences comprising the nucleotide sequence of the human Flk-1-intron;
   (d) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a), (b) or (c) under stringent conditions;
   (e) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a), (b) and (c); and

(f) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (e) capable of conferring expression in endothelial cells.

4. The recombinant DNA molecule of any one of claims 1 to 3, wherein said heterologous DNA sequence is operatively linked to further regulatory sequences.

5. The recombinant DNA molecule of claim 4, wherein said further regulatory sequence is a promoter.

6. The recombinant DNA molecule of claim 4 or 5, wherein said further regulatory sequence is a 3'-untranslated region.

7. The recombinant DNA molecule of claim 5 or 6, wherein said promoter is a promoter of hypoxia inducible genes, genes encoding growth factors or its receptors or glycolytic enzymes.

8. The recombinant DNA molecule of claim 7, wherein said growth factor is VEGF, PDGF or Fibroblast growth factor.

9. The recombinant DNA molecule of any one of claims 5 to 8, wherein said promoter comprises a DNA sequence selected from the group consisting of
   (a) DNA sequences comprising the nucleotide sequence as given in SEQ ID NO:1 from nucleotide 6036 to nucleotide 6959;
   (b) DNA sequences comprising the nucleotide sequence of the human Flk-1/KDR promoter;
   (c) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a) or (b) under stringent conditions;
   (d) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a) and (b); and

(e) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (d).

10. The recombinant DNA molecule of any one of claims 1 to 9, wherein at least one of said DNA sequences is of human or murine origin.

11. The recombinant DNA molecule of any one of claims 1 to 10, wherein said heterologous DNA sequence being operatively linked to said regulatory sequences is located 5' to said first regulatory sequence.

12. The recombinant DNA molecule of any one of claims 1 to 11, wherein said heterologous DNA sequence encodes a peptide, protein, antisense RNA, sense RNA and/or ribozyme.

13. The recombinant DNA molecule of claim 12, wherein said protein is selected from the group consisting of Vascular Endothelial Growth Factor (VEGF), Hypoxia Inducible Factors 7(HIF), HIF-Related Factor (HRF), tissue plasminogen activator, p21 cell cycle inhibitor, nitric oxide synthase, interferon-γ, atrial natriuretic polypeptide and monocyte chemotactic proteins.

14. The recombinant DNA molecule of claim 12, wherein said protein is a scorable marker, preferably luciferase, green fluorescent protein or lacZ.

15. The recombinant DNA molecule of claim 12, wherein said antisense RNA or said ribozyme are directed against a gene involved in vasculogenesis and/or angiogenesis and/or tumors of endothelial origin.

16. A nucleic acid molecule of at least 15 nucleotides in length hybridizing specifically with the first regulatory sequence of a recombinant DNA molecule of any one of claims 1 to 15.

17. A vector comprising a recombinant DNA molecule of any one of claims 1 to 15.

18. The vector of claim 17, which is an expression vector and/or a targeting vector.

19. The vector of claim 17 or 18, further comprising a gene capable of expressing HIF-2α.

20. A cell transformed with a DNA molecule of any one of claims 1 to 15 or the vector of any one of claims 17 to 19.

21. The cell of claim 20, which is a prokaryotic or eukaryotic cell.

22. The cell of claim 20 or 21, which is an endothelial cell.

23. The cell of any one of claims 20 to 22, further comprising a recombinant DNA molecule or vector containing a gene capable of expressing HIF-2α.

24. A pharmaceutical composition comprising a recombinant DNA molecule of any one of claims 1 to 15, the vector of any one of claims 17 to 19 and/or the nucleic acid molecule of claim 16 and optionally a pharmaceutically acceptable carrier.

25. A diagnostic composition comprising a recombinant DNA molecule of any one of claims 1 to 15, the vector of any one of claims 17 to 19, the cell of any one of claims 20 to 23 and/or the nucleic acid molecule of claim 16, and optionally suitable means for detection.

26. A method for the production of a transgenic non-human animal, comprising introduction of a recombinant DNA molecule of any one of claims 1 to 15 or a vector of any one of claims 17 to 19 into a germ cell, an embryonic cell or an egg cell or a cell derived therefrom.

27. A transgenic non-human animal comprising stably integrated into its genome a recombinant DNA molecule of any one of claims 1 to 15 and/or the vector of any one of claims 17 to 19 or obtained according to the method of claim 26.

28. The method of claim 26 or the transgenic non-human animal of claim 27, wherein said animal is a mouse.

29. A method for the identification of a chemical and/or biological substance capable of suppressing the transcription of a gene in endothelial cells comprising:
    (a) contacting a cell of any one of claims 20 to 23 or the transgenic non-human animal of claim 27 or 28 either of which is capable of expressing the heterologous DNA sequence with a plurality of compounds; and
    (b) determining those compounds which suppress the expression of said heterologous DNA sequence.

30. A method for the identification of a chemical and/or biological substance capable of activating and/or enhancing the transcription of a gene in endothelial cells comprising:
    (a) contacting a cell of any one of claims 20 to 23 or the transgenic non-human animal of claim 27 or 28 either of which is capable of expressing the heterologous DNA sequence with a plurality of compounds; and
    (b) determining those compounds which are capable of activating and/or enhancing the expression of said heterologous DNA sequence.

31. Use of a recombinant DNA molecule of any one of claims 1 to 15, the vector of any one of claims 17 to 19, the cell of any one of claims 20 to 23, the pharmaceutical composition of claim 24, the diagnostic composition of claim 25 and/or the transgenic non-human animal of claim 27 or 28 for the identification of a chemical and/or biological substance capable of suppressing or activating

and/or enhancing the transcription, expression and/or activity of genes and/or its expression products in endothelial cells.

32. The method of claim 29 or 30 or the use of claim 31, wherein the chemical and/or biological substance is selected from the group consisting of peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, neurotransmitters, peptidomimics and PNAs.

33. A method for the production of a pharmaceutical composition comprising the steps of the method of claim 29 or 30 and (c) formulating the compound identified in step (b) in a pharmaceutically acceptable form.

34. A method of inhibiting a vascular disease in a subject, comprising contacting an artery of said mammal with the vector of any one of claims 17 to 19, wherein said heterologous DNA sequence encodes a protein that reduces or prevents the development of the vascular disease.

35. The method of claim 34, wherein said protein reduces proliferation of smooth muscle cells.

36. Use of a recombinant DNA molecule of any one of claims 1 to 15, the vector of any one of claims 17 to 19, the nucleic acid molecule of claim 16 and/or a substance identified by the method of claims 29, 30 or 32 for the preparation of a composition for directing or preventing expression of genes specifically in endothelial cells.

37. Use of a recombinant DNA molecule of any one of claims 1 to 15, the vector of any one of claims 17 to 19, the nucleic acid molecule of claim 16 and/or a substance identified by the method of claims 29, 30 or 32 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying a vascular disease and/or a tumorous disease in a subject.

38. Use of a recombinant DNA molecule of any one of claims 1 to 15, the vector of any one of claims 17 to 19 and/or the nucleic acid molecule of claim 16 for the preparation of a pharmaceutical composition for inducing a vascular disease in a non-human animal or in the transgenic non-human animal of claim 27 or 28.

39. The method of claim 34 or 35 or the use of any one of claims 36 to 38, wherein the vascular disease is atherosclerosis and/or a neuronal disorder.

40. Use of a regulatory sequence as defined in any one of claims 1 to 3 for enhancing and/or directing gene expression in endothelial cells.

-6660 TCTAGAATAT AGAAGATAAG TTTGCGTACA ATTCAGTCCT TTGAAGACCT
GATAAGCTTT AAGAAGGAAG ATGGGTTACA CATTGGGAAA TGGTTGCAAT
CTGCACATGG CAGAGGCAAG AGATGCAAAT CACATTTCTT ACATACTCCA
-6510 TACAAATCTT ACAAGACTGT TTTTCTTTCT CATTTAAAAT AAGAAGACCT
GCCAGTCTTC CCCTTATTAC TAATTACAGT CACTCTGTAT CTTTGTTGAC
ATTGGATAGT TTTACATACT TCAACAGGCT GGTGTCATTA AAGTTGTGGT
GGGTGGGCAC CAGAGACACG TGATTCAGAG TGGGAGGAGA TGCAGGAGAA
ACGAGGCACA GCAGAAGCAG AAGCGAGGAA AAACACTCTC AACGTTACTA
ACACATCGAG AGGTTCCGCA CACTAGCAAT ACGGGCTGAA TCTGACCTAA
TCTCTGCTGT TGAAAATTTT GCCTAGCCGC ACACTAGCAA TACGGGCTGA
ATCTGACCTA ATCTCTGCTG TTGAAAATTT TGCCTAGCCT GTCACACAAG
TGCTGAGCAT ACAGAAAAAG GAGAGTAATT CTCTGGTTCT TTGACTAACC
AAATAGTCTA TATCAAATTG CCTAAGATAA TGTATACATT TAGTACATGA
-6010 CTGGTTATAC CTATTCTATA TGACTATTAT TTAAATGTGA ATTTACAAGT
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TTGGACATAG TTATATTTTT CCATTTATTT ATTTACTTTA TATCCTGATC
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CACTTGCCAC TTCTCCCATT TACTGATTTT ATCTGTGTGC AGACATTCAT
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TCCTTCTACC GTGTGAGTCC CTGGCCTCAA ACTCAGGTTG TCGGGCTTCA

Figure 1

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ATTCATCGTT ATCCCCTTTA CCAATTTTAC TATGTATTAA TTGCCACCCC

TTTAAATTTA ATTACTTCCT TGGCTGGGTT TTACAGGAGA GTTCCAGGAA

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AGGACCTAGG TTCAAGTCCT GGCACTCAGA GGTGGCTCAC AATCATCTGT

-5010 GACTTCAGTT CCAGGGGATC TGAAGAATTC TTCTGGGCTC CATGGGCATC

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TGGGGATGTA GTGATTTGTA CAAGAATGTC CCCTGTGGGC TCAGATATTT

GAATACTTAG TTCCCAGTTG GGGGAGCTTT TGTAGGGAGG TTGGGAGGCA

CAGCCTGGCA GGAGGAAGCA TGCTAGCAGC TTTGAGACTA TAAACCCTCA

TCTACTACCT TGTTCTCTTT CTGCATTGTG CTGTGTCTGA CACTGTGAGA

TTCCTGCTCC CGATGCCATG CCTGCCCGCC ATGATAGACT CCTAGCCCTC

TGGAAAGGTA ACCTCAGTGA ACTCTCTTCT ATAAGTTTCT TTGCTCCTGG

HindIII (-4200)

TGTTTTATCA CTGAAACGGA AAAGCTTGCA GGGAGGTAGG AGGCAGCCTG

Figure 1 continued

BstEII (-4100)

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CAAATCTTAT TTAGCAACTT AGAACTGTGA AATATTGGAA AGCTACTTAA
-4010 ACCTTCTAAA CTCCCTCCTC CACACTATGA GAATGTTACA TTTTCTATTC
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CAAGAGTGCT CCTAAATGGA CTTGCTTGTT ATTCATTTAC AGTGTGGCCC
CTTGACTTTC ATCGGCACTC CTAGCAGAAA ACAAAATCCG CCAGATGGAG
CTGGAGAGAT GGCTCAGCTG TTAAGAATAC TTATCCCTAC ACAGGCCCTG
GAGCCAGTTC CCAGCACCCA CACGGTGGCT CACAACCATC TGTAACTCCA
GTTCTAGGAG ACCCGACTCC CTCTTCTGTC TGAAAACACC AGGCACGCGT
GCGGTCTACA TACAAACATG AAAGCAAAAT ACACACATTA CATAAATAAA
TCTTAAAAAA TGATTCGGGG TGGGGGAAGG AAAAAAAAGG ATGTTAGAAA
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CAGATTTTCC AGAGCTGTAG AAATGGGCTG TGAGACGCCC ACTGTGGGTG
TTCGGAACCA AACTCGGGTC CTGTGGAAAG ACAGCGAGCA CCCATAATGC
AGAGGTATCT CTCAGATTTT ACTTTAAAAT TTCAATTTTC TTTTTTTTTT
TTAAAGTTCC AAGTAACTAT AGGAAAGTAC ATGGGTATAT AGATCCCCAG
-3010 TACCAAGATT CTTCCTTTGC AGGTAGCACA ACTTGGTTTG TTTCACATAA
AGAATGGAAA GTCATTAAAA CACTCATCAC ACTGTAAAGT AGAATTGAAC
TCTGACAGAA CAAGCGAAGT GAGTCTGACT TCCAGGTAAC TGAGCCTTCT

Figure 1 continued

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GGTGAGAAGG AAACAACGCA GGAGGGCTAG CCAAGTCTGA GAGTCGTGAG
TGTGCTCGGT TTATAAACGG AGCCCACCTT GCCAGCGAGG TAGTCACATG
CTCTGCTAAA CAGAAACTTA AGAAAACACT TACACGAAGC AAACATGGGG
AAGTGCCATG CAAGCATGTG ACTGACTGGT GGCAATGACC GAAACCACAG
CAGCCACTAG AAAAGGAAGG GTAGTGCGCC ACACTGTAGT TGTGAAAATG
AACTTATTCA TTTATTTTGA AAAACGTGTA AGAAGCAAAG ATGTCTTCTT
TCCCACCTAC CTTTGCGGCA GGCGAGCACT TCCTGGAATT TATAAAGTGC
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TGTCAAATAG AGAATGCTCT TGAACAAGTG TGTGTGTGTG TGTGTGTGCG
CGCGCACGCG CACTCACTCC TGCTCTGTTG AGGTCCAGTT TTGATGGTCC
CGCCAGAGGT ATATTTGAGT ATCATTTCTC AAGAGCTTCA GCTGGGAGAC
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-2010 TGTCTTTAAA AAAAAAAAAA GGCCCAAAAC AAGTGTAAAG TATTTCCCTA
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AGCCCTCCTT GGCTCTTTTG CTGAGGTTTG TTTGAGTTTG TTTTACTCTC
TGCAAGAGAA GTTTCCTTAA ACATTCTACC CTGTTCACAA GTAAATACAC
CTCTTAGCTA AGAGGCCACA CACCCAGGGG GAACACCGAT AAAAAGAACA

Figure 1 continued

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TTTTTATAGT GAACCTGGAT TTTACAAACC CTCCTGGTTT GCCACCTGCT

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TTCCCAGGTT GCTGGGGGCA GCAAGTGTCT CAGAGCCCAT TACTGGCTAC

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Figure 1 continued

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+1 (transcription start)

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GACTGTGTCC CGCAGCCGGG ATAACCTGGC TGACCCGATT CCGCGGACAC

CGCTGCAGCC GCGGCTGGAG CCAGGGCGCC GGTGCCCCGC GCTCTCCCCG

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VRE

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CGTGGAGACC CGAGCCGCCT CTGTGGGTAA GAAGCCCACT CTTTAGTAGT

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Figure 1 continued

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Figure 1 continued

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TGAACTTGGG AGATATTTTT CTTCCCCAAT TCCTTTTCCT CCATTTAAAT

GTGCTGTCTT TAGAAGCCAC TGCCTCAGCT TCTGCAGCTC AGATACCAAA

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GAGGCCTTTT AAAACTTGGA GTGTGCATTG TTTATGGAAG GGCTTTCCTA

BamHI (+3900)

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+3990 **TCCATCCCCC CAAGCTCAGC ACACAGAAAG ACATACTGAC AATTTTGGCA**

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TTTCCTATGT GAAAGGACTC ATTGTTTACT GAGGTCACAA CAATTTCCAC

TATTGCAGAA GTATAATAGT ATTGTTACAA TTGTTTATAA ATCATGAGAC

TTCTAAGAAC CTATTTAATA ATGAAACAAT GGAAAAAGTC TTTTCAAACC

TTTGTACTCT TTTGCTGAGC CGTTTTCAAC ATGCACAAAC ATATTACACA

AATATAACAT ACACAGGAAC ACACATGAAT GCATGGGATG ATGTGCCTAA

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Figure 1 continued

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Figure 1 continued

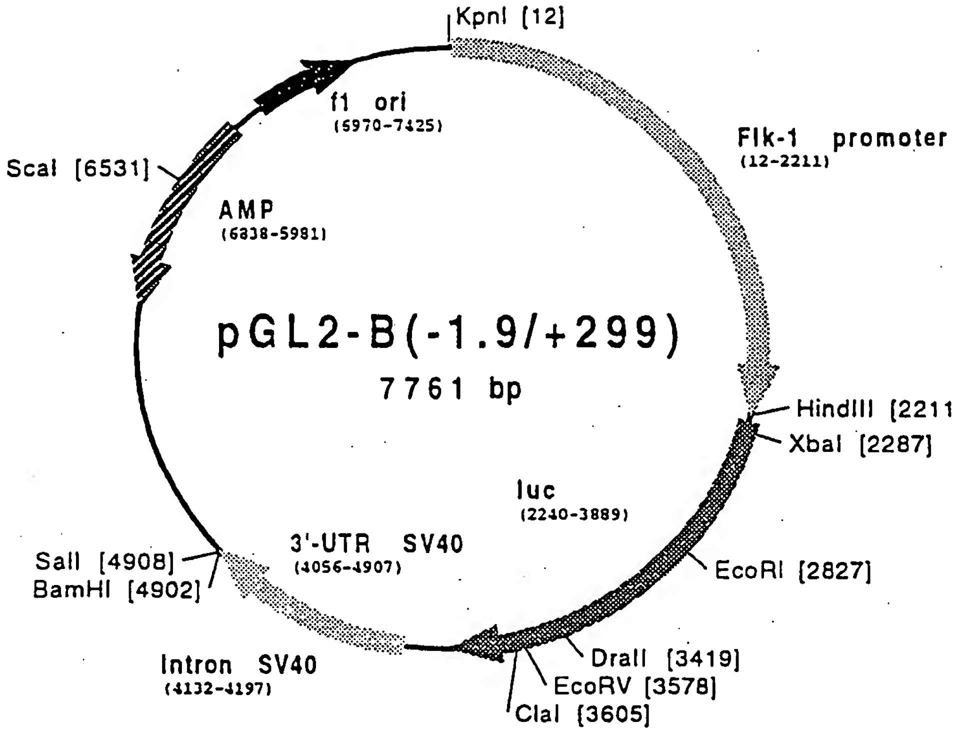

KpnI [12]
Flk-1 promoter
(12-2211)
HindIII [2211]
XbaI [2287]
luc
(2240-3889)
EcoRI [2827]
DraII [3419]
EcoRV [3578]
ClaI [3605]
Intron SV40
(4132-4197)
3'-UTR SV40
(4056-4907)
SalI [4908]
BamHI [4902]
ScaI [6531]
AMP
(6838-5981)
f1 ori
(5970-7425)
pGL2-B(-1.9/+299)
7761 bp

Figure 2

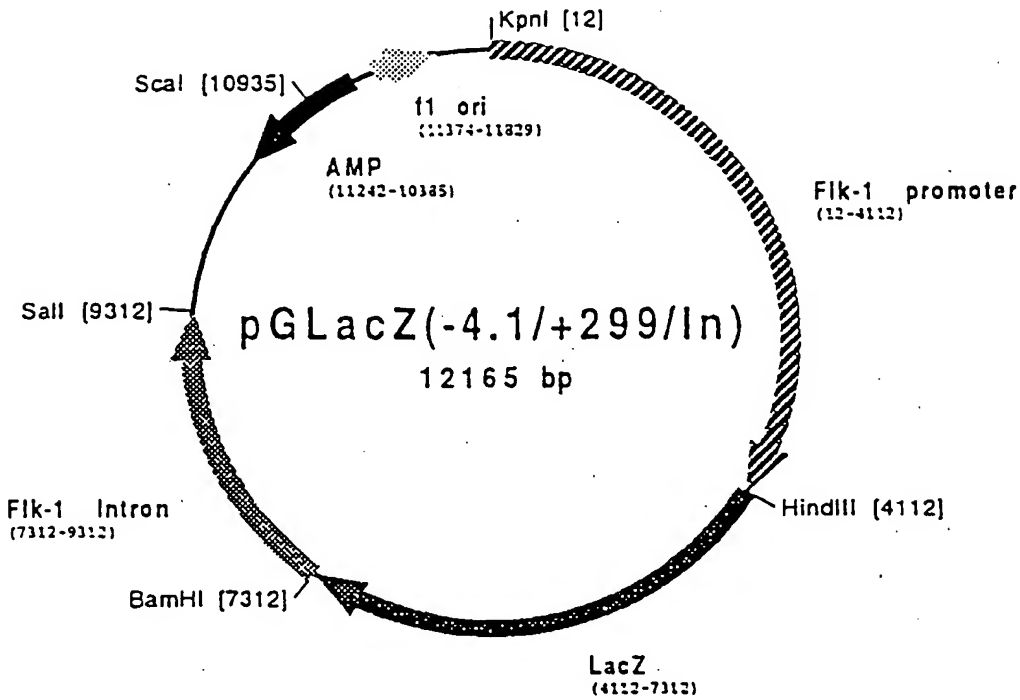

KpnI [12]
ScaI [10935]
f1 ori
(11374-11829)
AMP
(11242-10385)
Flk-1 promoter
(12-4112)
SalI [9312]
pGLacZ(-4.1/+299/In)
12165 bp
HindIII [4112]
Flk-1 Intron
(7312-9312)
BamHI [7312]
LacZ
(4112-7312)

Figure 3

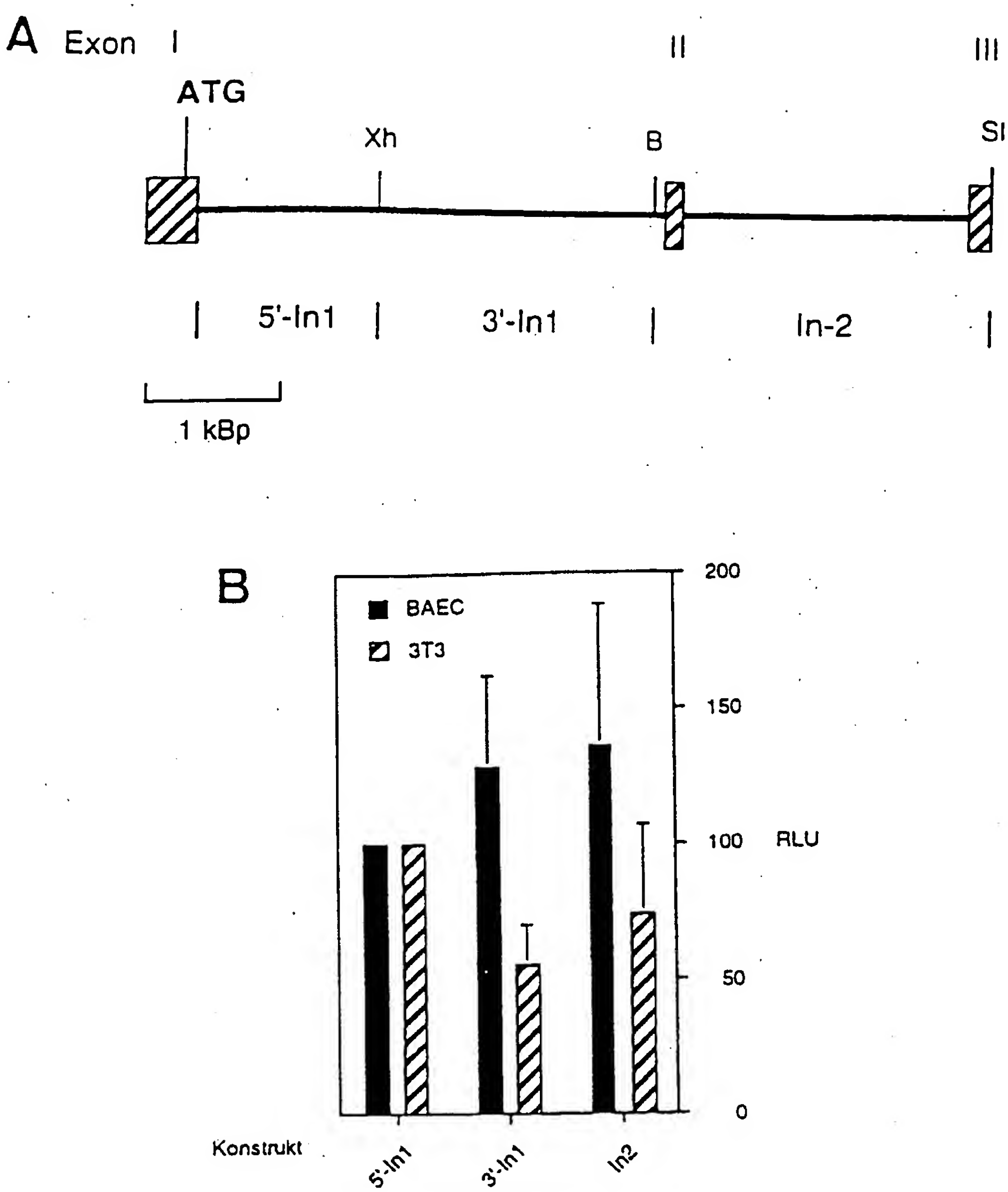

A
Exon I
II
III
ATG
Xh
B
SI
5'-In1
3'-In1
In-2
1 kBp
B
BAEC
3T3
200
150
100
50
0
RLU
Konstrukt
5'-In1
3'-In1
In2

Figure 4

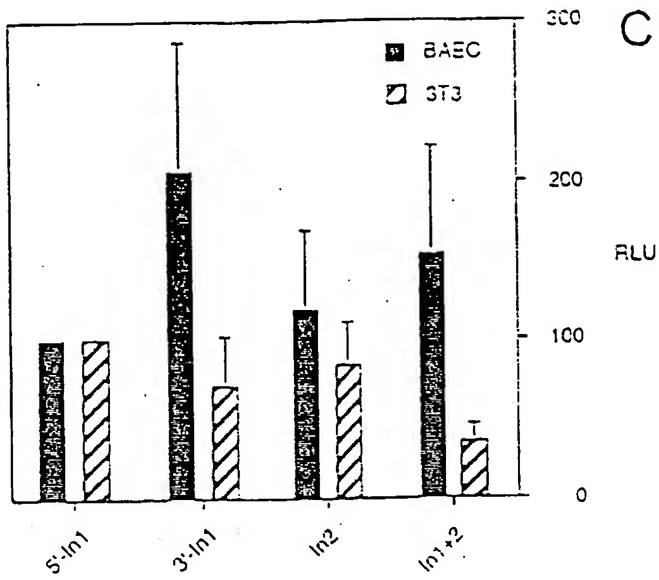


Figure 4 continued

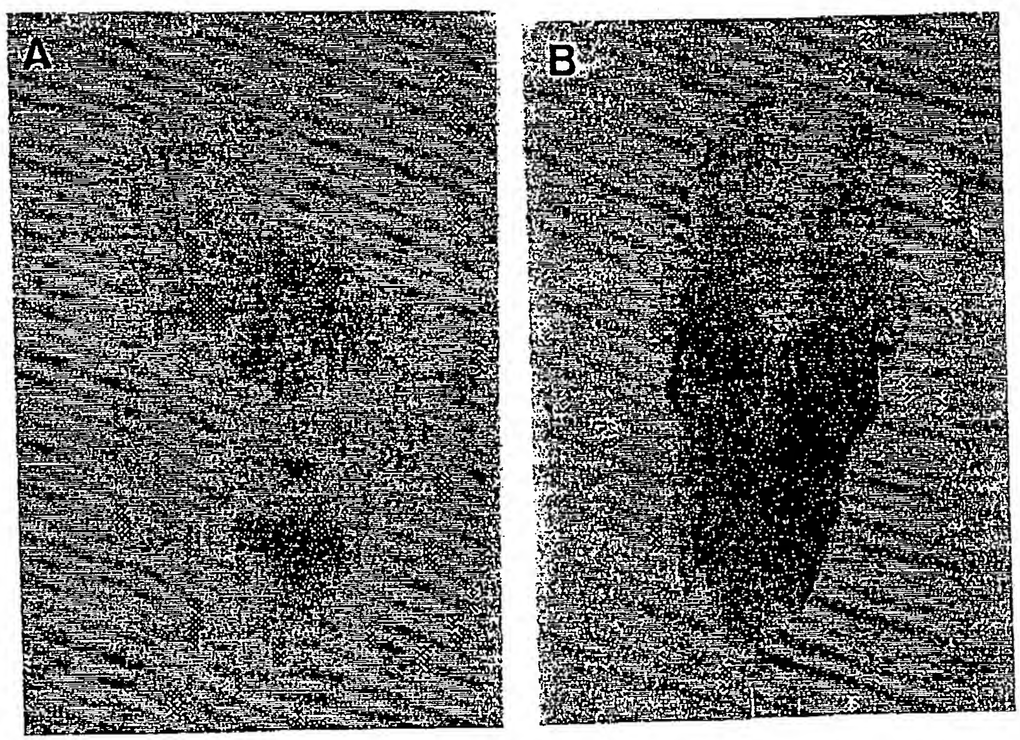


Figure 5

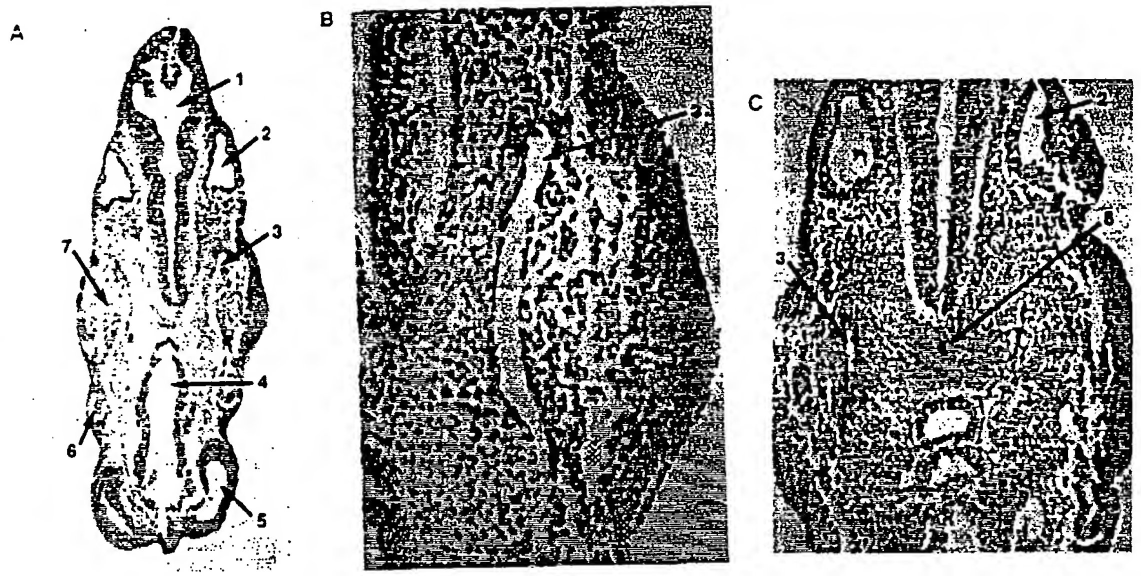


Figure 6

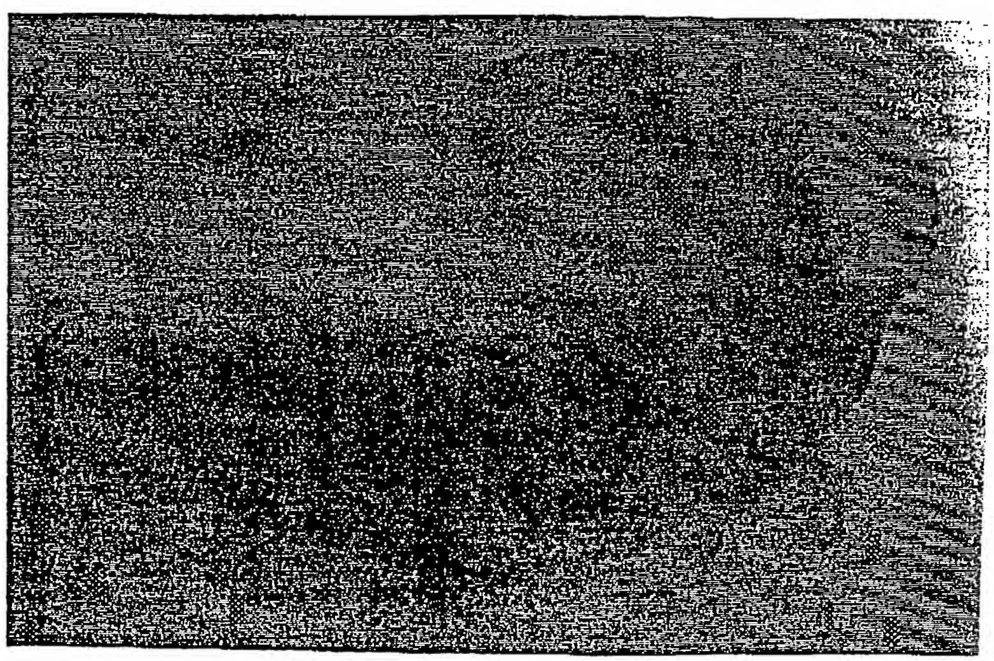
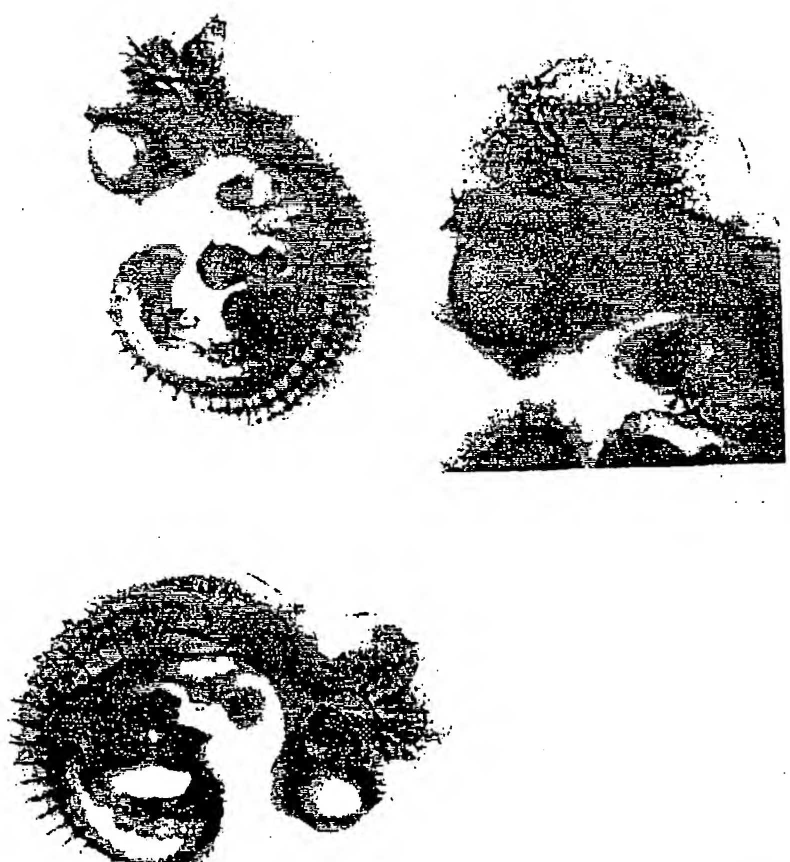


Figure 7

Figure 8

A B



C

Figure 9

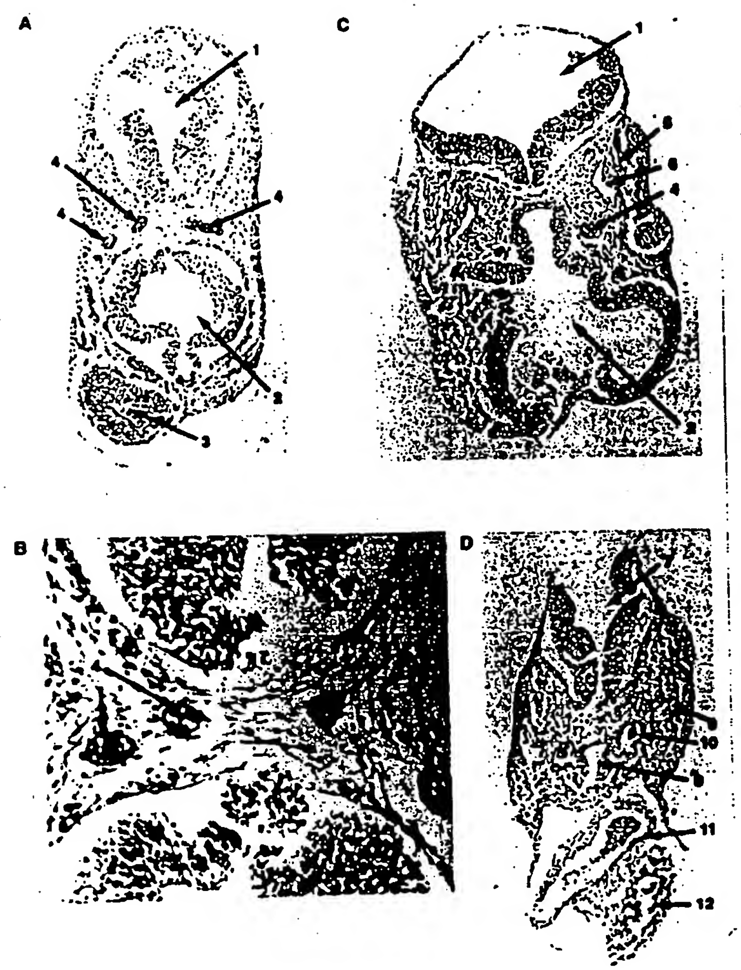


Figure 10

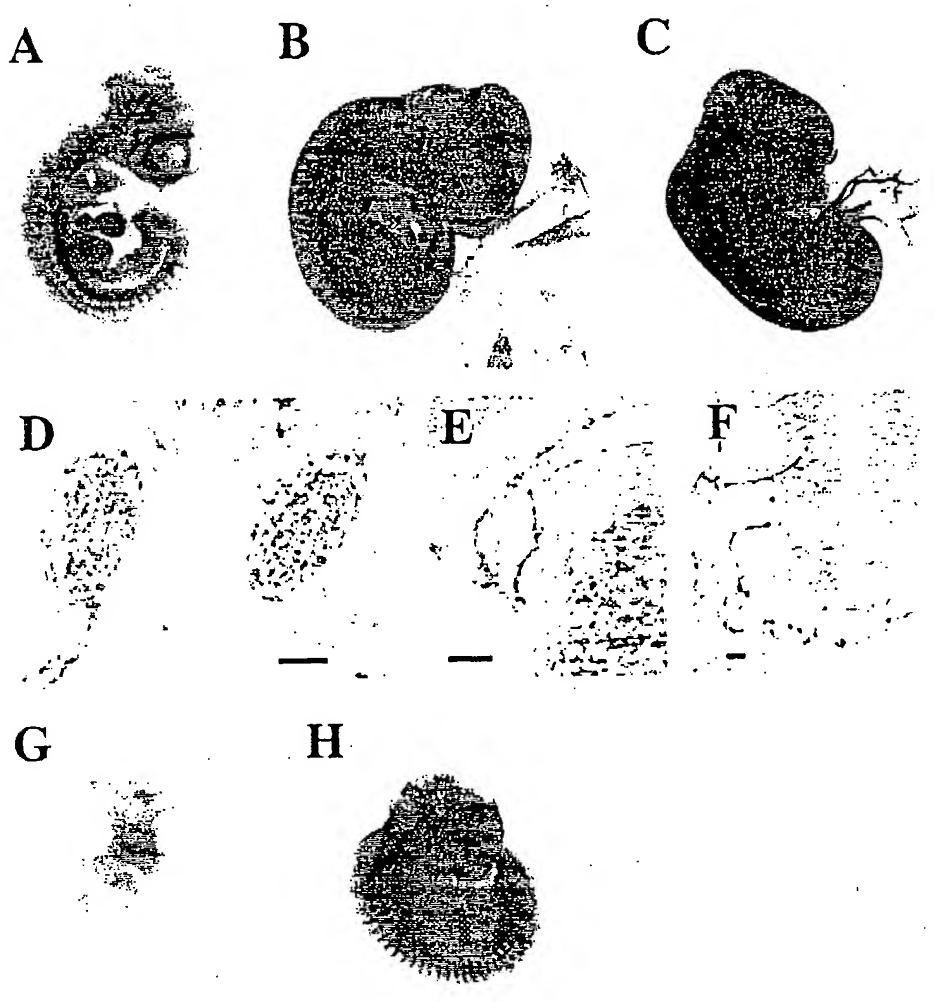


Figure 11

```
                                              GATA   PEA3
AAATGTGCTGTCTTTAGAAGCCACTGCCTCAGCTTCTGCAGCTCAGATACCAAAGGAAGTCTGGT    65
     GATA              AP1
ACACAGCATGATAAAAGACAATGGGACGGGGTCACAGTGGCTCCCGTCCCTTTCAGGGGTATGGA    130
                       NFkB                AP1
GACGAGCTGTAGAGAGATGTCTCCAGGGAGTTTTCATTAATCAGCAATTTAGTCAGATCTGTGCA    195
       STAT                      SCL/TAL-1
TCCTATGCTTTACAAGAAATGTCAGTGGGCCTGAGATCATCAGATGGAGGTTCATCGGGTTTCAA    260
 Ets-1 GATA                          Ets-1
TGTCCCGTATCCTTTTGTAAGACCTTGAAGTTGGCAACGCAGGAAAACAGGAACTCCACCCTGGT    325
                                 SCL/TAL-1 Ets-1
GCCGTGAATTGCAGAGCTGTTGTGTTGGTTTGTGACCATCTGCCCATTCTTCCTGTTATGACAGA    390

GCTTGTGAACTTTAACTGGGACTGGGGCAAAGTCAATCCCACCTTTATACAATGAATTGCTGAAG    455

AGGCCTTTTAAAACTTGGAGTGTGCATTGTTTATGGAAGGGCTTTCCTATTGGATC             511
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Figure 12

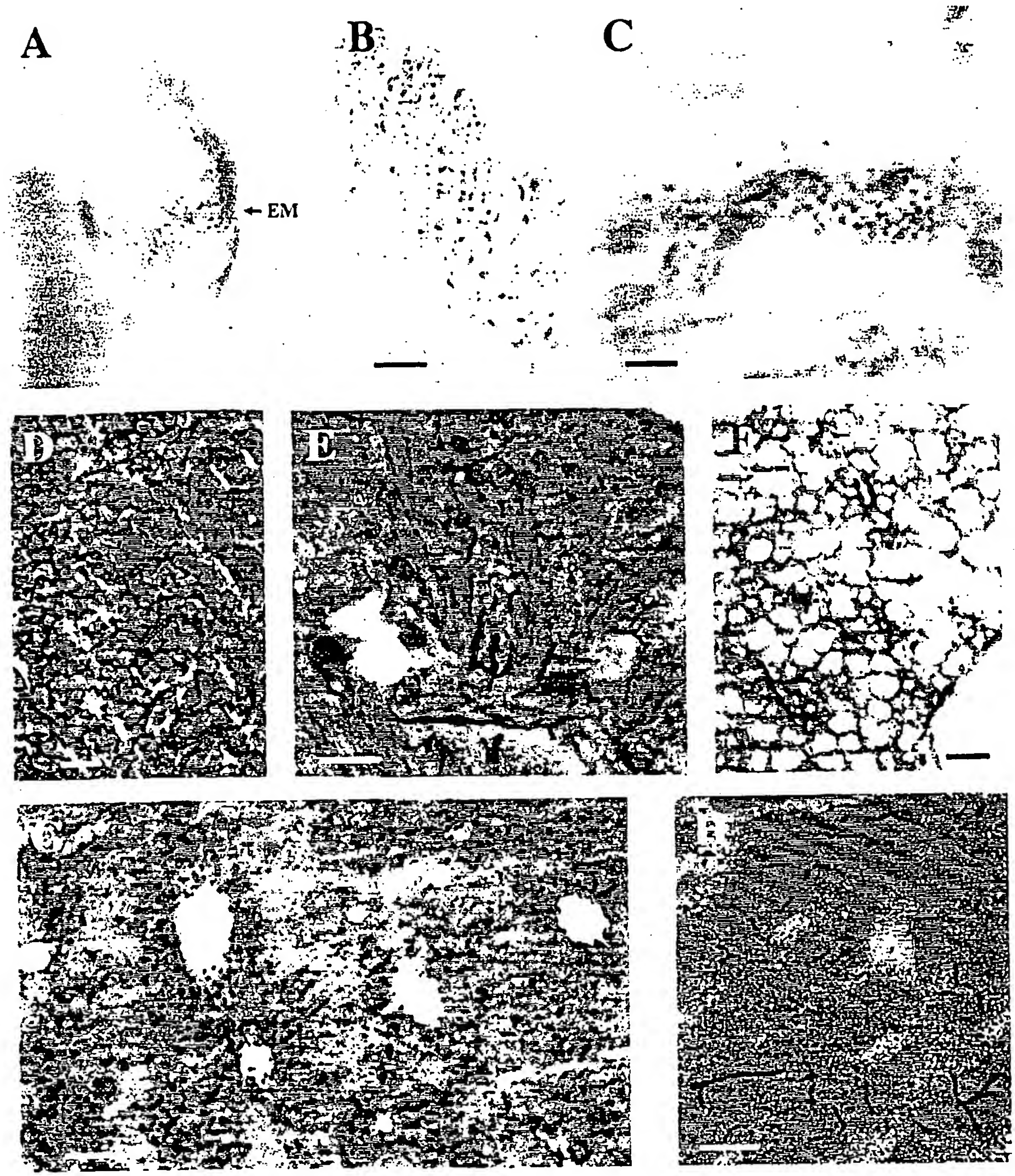

Figure 13

Figure 14

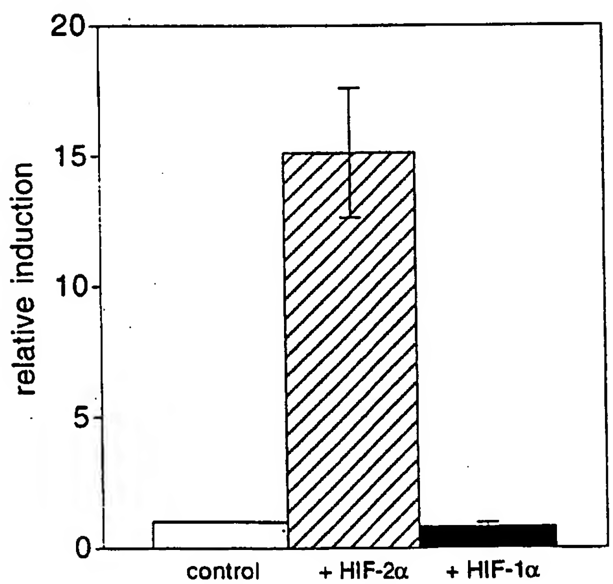

Figure 15

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/03318

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 C12N15/12 C12N15/19 C07K14/47 C07K14/52 C12N15/58 C12N9/72 C12N15/53 C12N9/02 C12N15/23 C07K14/57 C12N15/16 C07K14/58 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K C12Q A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 17359 A (REGENTS OF UNIVERSITY OF CALIFORNIA (US); WILLIAMS L.; MORISHITA K.) 15 May 1997 see abstract see page 2, line 32 - page 3, line 7 see page 45, line 4-31 see page 53 - page 59; claims ---	1,4,10, 12,14, 16-18, 20-22, 25,40

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1998	11/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/03318

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 A01K67/027 G01N33/50 A61K35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RÖNICKE V ET AL: "Characterization of the endothelium-specific murine vascular endothelial growht factor receptor-2 (Flk-1) promoter" CIRCULATION RESEARCH, vol. 79, no. 2, August 1996, pages 277-285, XP000604137 cited in the application see page 279, right-hand column, line 6-7 see page 280; figure 2 see page 282; figure 5B see page 283, left-hand column, line 3-9 --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1998	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SCHLAEGER T.M. ET AL.: "Uniform vascular-endothelial-cell -specific gene expression in both embryonic and adult transgenic mice" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, April 1997, pages 3058-3063, XP002081315 cited in the application see abstract ---	1,2
A	JORCYK C.L. ET AL.: "Multiple regulatory regions control the expression of Ets-1 in the developing mouse: vascular expression conferred by intron 1" CELLULAR AND MOLECULAR BIOLOGY, vol. 43, no. 2, March 1997, pages 211-225, XP002081894 cited in the application see abstract ---	1,2
A	PATTERSON C. ET AL.: "Cloning and functional analysis of the promoter for KDR/flk-1, a receptor for vascular endothelial growth factor" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 39, 29 September 1995, pages 23111-23118, XP002081230 cited in the application ---	
A	QUANDT K. ET AL.: "MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data" NUCLEIC ACIDS RESEARCH, vol. 23, no. 23, 1995, pages 4878-4884, XP002081231 cited in the application -----	

INTERNATIONAL SEARCH REPORT	International application No. PCT/EP 98/03318

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 34, 35 and 39 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/EP 98/03318

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9717359 A	15-05-1997	AU 7446696 A	29-05-1997

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00, C12N 15/09, 5/10, 5/00, 15/63	A1	(11) International Publication Number: WO 99/30743 (43) International Publication Date: 24 June 1999 (24.06.99)

(21) International Application Number: PCT/US98/08794

(22) International Filing Date: 30 April 1998 (30.04.98)

(30) Priority Data:
60/069,945 17 December 1997 (17.12.97) US

(71) Applicants: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Charlestown, MA 02110 (US). SHISEIDO CO., LTD. [–/JP]; 2–12–1, Fukuura, Kanazawa–ku, Yokohama–shi 103 (JP).

(72) Inventors: BURGESON, Robert, E.; 103 Appleton #4, Boston, MA 02116 (US). AMANO, Satoshi; 2–12–1, Fukuura, Kanazawa–ku, Yokohama–shi 103 (JP). KISHIMOTO, Jiro; 2–12–1, Fukuura, Kanazawa–ku, Yokohama–shi 103 (JP). NISHIYAMA, Toshio; 2–12–1, Fukuura, Kanazawa–ku, Yokohama–shi 103 (JP). EHAMA, Ritsuko; 2–12–1, Fukuura, Kanazawa–ku, Yokohama–shi 103 (JP).

(74) Agent: MYERS, Paul, Louis; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHODS FOR EVALUATING A COMPOUND FOR ITS EFFECT ON SKIN

(57) Abstract

The invention provides methods of evaluating a treatment for its effect on skin. The invention also provides non–human transgenic animals, e.g., mice, having a reporter gene coupled to a skin–metabolism promoter.

METHODS FOR EVALUATING A COMPOUND FOR ITS EFFECT ON SKIN

BACKGROUND OF THE INVENTION

The invention relates to transgenic animals which express reporter genes coupled to promoters of genes involved in the health, aging, or appearance of the skin, e.g., the versican, VEGF, or MMP promoters, and methods of using such animals in evaluating treatments, e.g., compounds, for their effect on skin.

SUMMARY OF THE INVENTION

The inventors have discovered that transgenic animals having one or more constructs which include a skin-metabolism promoter coupled to a reporter gene can be used to evaluate a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for use in enhancing the health or appearance of the skin.

Accordingly, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for its effect on skin. The method includes:

providing a transgenic animal having a reporter gene coupled to a skin metabolism-related promoter, preferably a human reporter;

administering the treatment to the transgenic animal or a tissue therefrom; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments, the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or transgenic mammal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In preferred embodiments, the promoter is heterologous from the transgenic animal, i.e., the promoter is from another species. In other preferred embodiments the promoter is from the same species as the transgenic animal. In particularly preferred embodiments, the skin metabolism-related promoter is a human skin metabolism-related promoter.

In particularly preferred embodiments, the skin metabolism-related promoter is: a promoter from a gene which encodes a transmembrane protein or a component of the extracellular matrix, such as a proteoglycan promoter, e.g., a versican promoter; a promoter from a protease expressed in the skin, e.g., a matrix metalloproteinase (MMP) promoter, e.g., an MMP1, MMP2, MMP3, MMP4, MMP5, MMP6, MMP7, MMP8, or MMP9 promoter; a promoter from a gene which affects vascular function, e.g., a vascular endothelial growth factor promoter; a hyaluronan synthase promoter, e.g., a hyaluronan synthase 1 promoter, a hyaluronan synthase 2 promoter, or a hyaluronan synthase 3 promoter; a promoter for a collagenase expressed in the skin, e.g., a MMP2 or MMP9, preferably a MMP9, promoter; or a neutrophil elastase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments, the treatment is administered repeatedly, prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the transgenic animal further includes a second reporter gene coupled to a second skin metabolism-related promoter, wherein the second skin metabolism-related promoter is different from the first skin metabolism-related promoter. The reporter gene coupled to the first promoter can be the same or different from the reporter gene coupled to the second promoter. For example, the transgenic animal can include: a first reporter gene coupled to the versican promoter and a second reporter gene coupled to the vascular endothelial growth factor promoter; a first reporter gene coupled to the versican promoter and a second reporter gene coupled to a hyaluronan synthase promoter; a first reporter gene coupled to the hyaluronan synthase promoter and a second reporter gene coupled to the vascular endothelial growth factor promoter; a first reporter gene coupled to a matrix metalloproteinase (MMP) promoter and a second reporter gene coupled to a MMP2 or MMP9, preferably MMP9, promoter; a first reporter gene coupled to a matrix metalloproteinase (MMP) promoter and a second reporter gene coupled to the neutrophil elastase promoter; or a first reporter gene coupled to a MMP2 or MMP9, preferably a MMP9, promoter and a second reporter gene coupled to the neutrophil elastase promoter. In preferred embodiments, the transgenic animal can include two constructs both of which are upregulated. In preferred embodiments, the transgenic animal can include two constructs both of which are downregulated.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a second treatment) to the transgenic animal. The second treatment can be one which injures or damages the skin, kills skin cells, or can include the removal of hair, e.g., by plucking, shaving, or application of a depilatory, or in general, induces an unwanted condition of the skin. The second treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the treatment can be determined in the presence of the second treatment, and optionally compared to the response seen in the absence of the second treatment.

In another aspect, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound for its effect on skin. The method includes:

providing a transgenic animal, e.g., a mouse, having a reporter gene coupled to a, preferably human, versican promoter;

administering a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, to the transgenic animal, or to a tissue taken therefrom; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin aging.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or dead transgenic animal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the versican promoter is a human versican promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments the treatment is administered repeatedly, preferably prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a second treatment) to the transgenic animal. The second treatment can be one which injures or damages the skin, kills skin cells, or can include the removal of hair, e.g., by plucking, shaving, or application of a depilatory, or in general, induces an unwanted condition of the skin. The second treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the treatment can be determined in the presence of the second treatment, and optionally compared to the response seen in the absence of the second treatment.

In another aspect, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for its effect on skin. The method includes:

providing a transgenic animal, e.g., a mouse, having a reporter gene coupled to a, preferably human, matrix metalloproteinase promoter;

administering the treatment to the transgenic animal, or to a tissue taken therefrom; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin aging.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or dead transgenic animal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of

the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the matrix metalloproteinase promoter is a human matrix metalloproteinase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments, the treatment is administered repeatedly, preferably prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a second treatment to the transgenic animal. The second treatment can be one which injures or damages the skin, kills skin cells, or can include the removal of hair, e.g., by plucking, shaving, or application of a depilatory, or in general, induces an unwanted condition of the skin. The second treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the treatment can be determined in the presence of the second treatment, and optionally compared to the response seen in the absence of the second treatment.

In another aspect, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for its effect on skin. The method includes:

providing a transgenic animal, e.g. a mouse, having a reporter gene coupled to a vascular endothelial growth factor promoter;

administering the treatment to the transgenic animal; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin aging.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or dead transgenic animal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering

the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the vascular endothelial growth factor promoter is a human vascular endothelial growth factor promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments, the treatment is administered repeatedly, preferably prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a second treatment to the transgenic animal. The second treatment can be one which injures or damages the skin, kills skin cells, or can include the removal of hair, e.g., by plucking, shaving, or application of a depilatory, or in general, induces an unwanted condition of the skin. The second treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the treatment can be determined in the presence of the second treatment, and optionally compared to the response seen in the absence of the second treatment.

In another aspect, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for its effect on skin. The method includes:

providing a transgenic animal, e.g., a mouse, having a reporter gene coupled to a, preferably human, hyaluronan synthase promoter;

administering the treatment to the transgenic animal; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin aging.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or dead transgenic animal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle,

for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the hyaluronan synthase promoter is a human hyaluronan synthase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments, the treatment is administered repeatedly, preferably prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a second treatment to the transgenic animal. The second treatment can be one which injures or

damages the skin, kills skin cells, or can include the removal of hair, e.g., by plucking, shaving, or application of a depilatory, or in general, induces an unwanted condition of the skin. The second treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the treatment can be determined in the presence of the second treatment, and optionally compared to the response seen in the absence of the second treatment.

In another aspect, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for its effect on skin. The method includes:

providing a transgenic animal, e.g., a mouse, having a reporter gene coupled to, a preferably human, MMP2 or MMP9, preferably a MMP9, promoter;

administering the treatment to the transgenic animal, or to a tissue taken therefrom; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin aging.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or dead transgenic animal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, a MMP2 or MMP9, preferably a MMP9, promoter is a human MMP2 or MMP9, preferably a MMP9, promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments, the treatment is administered repeatedly, preferably prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a second treatment to the transgenic animal. The second treatment can be one which injures or damages the skin, kills skin cells, or can include the removal of hair, e.g., by plucking, shaving, or application of a depilatory, or in general, induces an unwanted condition of

the skin. The second treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the treatment can be determined in the presence of the second treatment, and optionally compared to the response seen in the absence of the second treatment.

In another aspect, the invention features, a method of evaluating a treatment, e.g., the removal of hair, e.g., by plucking or shaving, or the administration of a compound, for its effect on skin. The method includes:

providing a transgenic animal, e.g., a mouse, having a reporter gene coupled to a, preferably human, neutrophil elastase promoter;

administering the treatment to the transgenic animal, or to a tissue taken therefrom; and

evaluating expression of the reporter gene, thereby evaluating the treatment for its effect on skin aging.

The treatment, e.g., the administration of a compound, can be administered to a live animal. In other embodiments the treatment, e.g., the administration of a compound, is administered to a tissue, e.g., a cell, taken from a transgenic animal.

The effect of the treatment, e.g., the administration of a compound, can be evaluated in a living transgenic animal, a dead transgenic animal, or tissue taken from either a living or dead transgenic animal.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

In preferred embodiments, the treatment includes the administration of a compound and the compound is administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic

guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the neutrophil elastase promoter is a human neutrophil elastase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In preferred embodiments, the treatment is administered repeatedly, preferably prior to evaluation of reporter gene evaluation.

In preferred embodiments, the treatment includes the administration of a compound and the method further includes one or more subsequent administrations of the compound to the transgenic animal. In preferred embodiments, the compound is administered to the transgenic animal for a period of at least one, two, three, or four weeks. The compound can be administered at a constant level or at a range of different levels. In preferred embodiments, the compound is administered to the transgenic animal before, during, or after UV irradiation or other skin damaging treatment.

In preferred embodiments, the method further includes comparing the expression of the reporter gene to a control value, e.g., the level of expression of the reported gene in an untreated transgenic animal.

In preferred embodiments, the method further includes evaluating the expression of the reporter gene coupled to the second skin metabolism-related promoter.

In preferred embodiments, the compound is: a cosmetic; a non-toxic substance; a substance approved for human drug or cosmetic use in one or more jurisdictions; a retinoid or derivative thereof; TGFβ; of TGFα.

In preferred embodiments, the method further includes administering a treatment (other than the compound) to the transgenic animal. The treatment can be one which injures or damages the skin, kills skin cells, or in general, induces an unwanted condition of the skin. The treatment can be the application of water, a drying agent, an irritant, an inflammatory agent, light or UV irradiation. Reporter gene expression in response to the

compound can be determined in the presence of the treatment, and optionally compared to the response seen in the absence of the treatment.

In another aspect, the invention features, a non-human transgenic animal described herein, e.g., a transgenic animal having a reporter gene coupled to a versican promoter.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the versican promoter is a human versican promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In another aspect, the invention features, a non-human transgenic animal, e.g., a mouse, or a tissue taken therefrom, having a reporter gene coupled to a matrix metalloproteinase promoter.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the matrix metalloproteinase promoter is a human matrix metalloproteinase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline

phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In another aspect, the invention features, a non-human transgenic animal, e.g., a mouse, or a tissue taken therefrom, having a reporter gene coupled to a vascular endothelial growth factor promoter.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the vascular endothelial growth factor promoter is a human vascular endothelial growth factor promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In another aspect, the invention features, a non-human transgenic animal, e.g., a mouse, or a tissue taken therefrom, having a reporter gene coupled to a hyaluronan synthase promoter.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, .g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the hyaluronan synthase promoter is a human hyaluronan synthase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline

phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In another aspect, the invention features, a non-human transgenic animal, e.g., a mouse, or a tissue taken therefrom, having a reporter gene coupled to a Type IV collagenase promoter.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, a MMP2 or MMP9, preferably a MMP9, promoter is a human a MMP2 or MMP9, preferably a MMP9, promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In another aspect, the invention features, a non-human transgenic animal, e.g., a mouse, or a tissue taken therefrom, having a reporter gene coupled to a neutrophil elastase promoter.

In preferred embodiments, the transgenic animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In particularly preferred embodiments, the neutrophil elastase promoter is a human neutrophil elastase promoter.

In preferred embodiments the reporter gene encodes a product which can be detected with relative ease, e.g., an enzyme, e.g., an enzyme which produces a colored or luminescent product. In particularly preferred embodiments, the reporter gene can be a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline

phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene.

In another aspect, the invention features a promoter-reporter gene construct described herein.

In another aspect, the invention features a method of analyzing GFP presence or distribution in a tissue. The method includes:

providing a tissue sample, e.g., a tissue section, which includes GFP;

evaluating or detecting fluorescent emission, or the lack of fluorescent emission, wherein said detecting step is performed prior to washing or fixing with an aqueous solution,

thereby analyzing GFP in a tissue.

In preferred embodiments the tissue is frozen prior to the detection step. The sample is not contacted with a fixing agent prior to detection.

In preferred embodiments: the tissue is from a transgenic animal, e.g., a transgenic mini-pig, guinea pig, rat or mouse, e.g., a hairless or nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging; the GFP is expressed from a transgenic sequence encoding GFP or under the control of a transgenic control element, e.g., a transgenic promoter or enhancer.

In preferred embodiments the promoter is a human promoter.

In preferred embodiments detection includes examination of the sample with a microscope, e.g., a fluorescent or epi-fluorescent microscope.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

Animals described herein can be used in this method.

In another aspect, the invention features, a method of determining the stage of the hair cycle in an animal which expresses a reporter molecule in hair follicle, e.g., the outer root sheath of the hair follicle. The method includes evaluating or detecting the presence or absence of reporter expression, presence being associated with a growing hair cycle (anagen) and absence with a resting hair cycle (telegen and catogen).

In preferred embodiments the reporter molecule is under the control of a promoter expressed in the hair follicle, e.g., the outer root sheath.

In preferred embodiments the promoter is a human promoter.

In preferred embodiments the reporter molecule is GFP.

In preferred embodiments the animal is a transgenic animal, e.g., a transgenic mini-pig, guinea pig, rat or mouse, e.g., a hairless or nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging;

Animals described herein can be used in this method.

In preferred embodiments the promoter is a VEGF promoter, a versican promoter, or other promoter described herein.

In another aspect, the invention features, a method of analyzing wound healing. The method includes: providing an animal which expresses a reporter molecule under the control of a VEGF promoter, detecting the presence or absence of reporter molecule in a wound, thereby analyzing wound healing.

In preferred embodiments the detection step is reported.

In preferred embodiments, the animal, tissue from the animal, is subjected to a treatment, e.g., the administration of a compound. In such embodiments the method can be used to evaluate the effect of the treatment on wound healing. It may be desirable to compare results from a treated subject or tissue with an untreated subject or tissue.

In preferred embodiments the reporter molecule is GFP.

In preferred embodiments the animal is a transgenic animal, e.g., a transgenic mini-pig, guinea pig, rat or mouse, e.g., a hairless or nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging.

Animals described herein can be used in this method.

In preferred embodiments the promoter is a human promoter.

In another aspect, the invention features, a method of analyzing GFP expression in a transgenic animal having a GFP transgene. The method includes:

a first step of evaluating or detecting the presence or absence of GFP in the animal or in a tissue from the animal; and (optionally)

a second step of evaluating or detecting the presence or absence of GFP in the animal or in a tissue from the animal; and

thereby analyzing GFP expression.

In preferred embodiments (in this method and in other methods disclosed herein) the GFP is red shifted GFP.

In preferred embodiments the animal is a transgenic animal, e.g., a transgenic mini-pig, guinea pig, rat or mouse, e.g., a hairless or nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919,

or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. An animal described herein can be used in this method.

In preferred embodiments at least 1, 5, 10, 20, 30, or 60, 180, 365 days elapse between first and second step.

In preferred embodiments the tissue is skin tissue.

In preferred embodiments the detection steps are performed on a live animal.

In preferred embodiments the promoter is a human promoter.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In another aspect, the invention features, a method of analyzing the expression of a transgene on a transgenic animal, e.g., a transgenic mouse or pig. The method includes:

providing a live transgenic animal;

evaluating or detecting the presence or absence of a reporter gene, e.g., GFP, encoded by a transgenic sequence or under the control of a transgenic control element, e.g., a promoter or enhancer;

thereby analyzing the expression of a transgene.

In preferred embodiments the animal is a transgenic animal, e.g., a transgenic mini-pig, guinea pig, rat or mouse, e.g., a hairless or nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. Animals described herein can be used in the methods.

In preferred embodiments the reporter is under the control of a VEGF promoter or another promoter described herein.

In preferred embodiments the promoter is a human promoter.

In preferred embodiments the promoter is one which is expressed on the skin.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the tissue is skin tissue.

In preferred embodiments the detection steps are performed on a live animal.

In preferred embodiments the method is repeated at least once during the life of the animal. The first and a subsequent repetition of the method can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days.

In another aspect, the invention features, a method of evaluating gene expression in a live animal. The method includes:

providing a live transgenic animal having a transgene which includes a reporter gene, e.g., GFP, e.g., red shifted GFP;

evaluating or detecting the presence or absence of reporter molecule, e.g., GFP, in the live transgenic animal, thereby evaluating gene expression in the live animal.

In preferred embodiments, the sequence which encodes a reporter gene is under the control of a presclected promoter, e.g., a human promoter. The preselected promoter can be, a skin metabolism-related promoter, e.g., : a promoter from a gene which encodes a transmembrane protein or a component of the extracellular matrix, such as a proteoglycan promoter, e.g., a versican promoter; a promoter from a protease expressed in the skin, e.g., a matrix metalloproteinase (MMP) promoter, e.g., an MMP1, MMP2, MMP3, MMP4, MMP5, MMP6, MMP7, MMP8, or MMP9 promoter; a promoter from a gene which affects vascular function, e.g., a vascular endothelial growth factor promoter; a hyaluronan synthase promoter, e.g., a hyaluronan synthase 1 promoter, a hyaluronan synthase 2 promoter, or a hyaluronan synthase 3 promoter; a promoter for a collagenase expressed in the skin, e.g., a MMP2 or MMP9, preferably a MMP9, promoter; or a neutrophil elastase promoter.

). The VEGF promoter is a preferred promoter. In preferred embodiments the promoter is one which is up or down regulated in inflammatory angiogenesis, or neoplastic growth.

In preferred embodiments, the animal is a non-human transgenic animal. For example, the transgenic animal can be a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, or a transgenic mouse, e.g., a hairless mouse, a nude mouse, a senescence accelerated mouse, e.g., SAM mice, see Takeda et al., 1991, *L. Am. Geriatr.* 39:911-919, or a transgenic mutant mouse which exhibits a phenotype of accelerated aging. The most preferred animals are mice.

In preferred embodiments, a treatment is administered to the animal any of before, during, or after valuation of reporter gene expression. The treatment can be the administration of a compound. The compound can be administered by: applying the compound to the skin of the transgenic animal; systemically administering the compound; orally administering the compound; or injecting the compound, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments evaluating includes detection of a signal, e.g., a fluorescent signal, with a confocal microscope.

In preferred embodiments the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal. The first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days. Both the first and a subsequent repetition can be performed on a live animal, e.g., with the use of a confocal microscope.

Methods of the invention can be performed *in vivo*, with whole animals, or *in vitro*, that is, with tissue, e.g., skin, or cells, which are derived from a transgenic animal described herein or with cells, preferably skin cells or tissue, from cells transformed with a skin-metabolism promoter/reporter gene construct.

As used herein, a "transgenic animal" is an animal, e.g., a non-human mammal, e.g., a mini-pig, a guinea-pig, or a rodent, e.g., a mouse or a rat, in which one or more, and preferably essentially all, of the cells of the animal include a transgene. The transgene can be introduced into the cell, directly or indirectly by introduction into a precursor of the cell, e.g., by microinjection, transfection or infection, e.g., by infection with a recombinant virus. The term genetic manipulation is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA.

Transgenic animals can be, e.g., heterozygous or homozygous for a transgene.

As used herein, the term "rodent" refers to all members of the phylogenetic order *Rodentia*.

As used herein, the term "reporter gene" refers to a nucleic acid sequence which is fused downstream of a skin metabolism-related promoter, such that its expression is under the control of the promoter. Reporter genes usually encode a protein whose activity can be easily measured. For example, the reporter gene can be a gene encoding an assayable enzyme not found in the cell in nature, e.g., a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, a chloramphenicol acetyl transferase gene, luciferase, and the like.

As used herein, the term "skin metabolism-related promoter" refers to a promoter which is transcriptionally active in the skin. It need not be skin-specific. The gene in which the promoter is naturally found can be a gene involved in the maintenance, or proper functioning of the skin. For example, the gene can be a gene encoding a protein which is part of the extracellular matrix, a protein involved in the maintenance or

degradation of the extracellular matrix, or a protein involved in supplying nutrition to the skin.

As used herein, "administering a compound to an animal" refers to dispensing, delivering or applying a treatment to an animal or cell. Administration can be by topical administration, by parenteral or oral route, intramuscular injection, subcutaneous/intradermal injection, intravenous injection, buccal administration, transdermal delivery or administration by the intranasal or respiratory tract route. The most preferred administrations are topical application or subcutaneous or intradermal injection.

The methods of the invention allow rapid and efficient evaluation of compounds for their effect on skin.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DETAILED DESCRIPTION

The drawings are first briefly described.

Brief Description of the Drawings

Figure 1 is a schematic representation of the human versican-Lac Z transgene construction.

Figure 2 is a schematic representation of the vascular endothelial growth factor-green fluorescent protein [VEGF-GFP] transgenic construct.

Figure 3 is a depiction of the nucleotide sequence of the 5069 pb MMP9 promoter-GFP construct.

Figure 4 is a depiction of the nucleotide sequence of the 7383 pb MMP9 promoter-beta-Gal construct.

Promoters

Methods of the invention allow evaluating a compound for its effect on the skin. Methods of the invention can be used to evaluate a compound for its effect on the health or appearance of the skin, e.g., for use as a cosmetic. The effect on skin is usually determined as an effect on the expression of a gene under the control of a skin-metabolism-related promoter. Such promoters include those which control the expression of: a product which is a component of the skin, e.g., the dermis or epidermis; a product which affects hydration or nutrition of the skin; a product which promotes the synthesis, or degradation, of components of the skin; a product which affects the

vasculature of the skin; a product which affects hair follicle metabolism; a product which affects skin glandular structures; a product which affects subcutaneous musculature; a product which affects adipose tissue; or a product which affects cutaneous nerves.

Methods of the invention are useful for evaluating a compound for an effect on a parameter related to the appearance or health of the skin, for example, the elasticity of the skin, the propensity of the skin to wrinkle, the ability of the skin to retain fluids, e.g., water or an oil, the ability of the skin to resist or repair damage, e.g., light or UV induced damage, the metabolism of hair follicles including growth cycling or pigment deposition, subcutaneous muscle tone and function, or neurotransmission by cutaneous nerves. Generally, effects on these parameters will be evaluated indirectly, e.g., by the effect on the expression of a reporter gene under the control of a promoter which is normally coupled to a gene which encodes a product which affects any of the these parameters.

Examples of such skin-metabolism-related promoters include a versican promoter; a matrix metalloproteinase promoter; a vascular endothelial growth factor promoter; a hyaluronan synthase promoter; a MMP2 or MMP9, preferably a MMP9, promoter; and a neutrophil elastase promoter.

The versican promoter regulates the expression of the versican gene, described in Naso M. F. et al. (1994) *J. Biol. Chem.* 269(52): 32999-33008, the contents of which are incorporated herein by reference. Versican is a large modular chondroitin sulfate proteoglycan expressed in the dermis and the epidermis of the skin. Versican can bind large amounts of water while remaining attached to the extracellular matrix and can, therefore, hydrate and fill the skin. Accordingly, compounds which result in *upregulation* of this gene are preferred.

A matrix metalloproteinase promoter regulates the expression of a matrix metalloproteinase gene. The matrix metalloproteinases (MMPs) belong to a family of extracellular matrix proteases, described in Mauch C. et al. (1994) *Arch. Dermatol. Res.* 287:107-114. As the name implies, these matrix metalloproteinases are involved in the degradation of the extracellular matrix in, for example, the dermis and the epidermis of the skin. Accordingly, compounds which result in *downregulation* of this gene are preferred.

The vascular endothelial growth factor promoter regulates the expression of the vascular endothelial growth factor gene described in, for example, Tischer E. (1991) *J. Biol. Chem.* 266(18): 11947-11954, the contents of which are incorporated herein by reference. The vascular endothelial growth factor is a mitogen for vascular endothelial

cells and, as a result, it can lead to proliferation of the microvasculature beneath the skin and increased vascular permeability. Increased microvasculature and vascular permeability allow for better nutrition (e.g., better nutrient delivery to the dermis and the epidermis) and hydration of the skin. Accordingly, compounds which result in *upregulation* of this gene are preferred.

The hyaluronan synthase (HAS1-3) promoters regulate the expression of the hyaluronan synthase (HAS1-3) genes, described in Itano N. et al. (1996) *BBRC* 222: 816-820; Watanabe K. (1996) *J. Biol. Chem.* 271(38): 22945-22948; and Spicer A. P. (1997) *J. Biol. Chem.* 272(14): 8957-8961, respectively, the contents of which are incorporated herein by reference. Hyaluronan synthases, as the name implies, are enzymes involved in the synthesis of hyaluronan, a linear unbranched glycosaminoglycan. Hyaluronan binds versican and generally acts as an anchor for other proteoglycans, leading to the stabilization of the proteoglycan "network." As a result, hyaluronan (like versican) can assist in the hydration and filling of the skin. Accordingly, compounds which result in *upregulation* of this gene are preferred.

The type IV collagenase promoter regulates the expression of the type IV collagenase gene, described in Huhtala P. (1991) *J. Biol. Chem.* 266(25): 16485-16490, the contents of which are incorporated herein by reference. Type IV collagenase is another member of the extracellular matrix protease family and it is involved in the degradation of various components of the extracellular matrix in, for example, the dermis and the epidermis of the skin. Accordingly, compounds which result in *downgulation* of this gene are preferred.

The neutrophil elastase promoter regulates the expression of the neutrophil elastase gene, described in Takahashi H. (1988) *J. Biol. Chem.* 263(29): 14739-14747, the contents of which are incorporated herein by reference. Neutrophil elastase is a powerful serine protease capable of cleaving most protein components of the extracellular matrix (including elastin) in, for example, the dermis and the epidermis of the skin. Accordingly, compounds which result in *downgulation* of this gene are preferred.

<u>Transgenic animals</u>

Transgenic animals which can be used in the methods of the invention include non-human mammals, such as pigs, e.g., mini-pigs, or guinea-pigs; or rodents, e.g., mice or rats, e.g., hairless mice (described in, for example, Begona M. et al. (1994) *Proc. Natl. Acad. Sci.* 91:7717-7721), nude mice, senescence accelerated mice (described in, for example, Takeda et al. (1991) *L. Am. Geriatr. Soc.* 39:911-19), or transgenic mutant

mice which exhibit a phenotype of accelerated aging; in which one or more, and preferably essentially all, of the cells of the animal include a transgene. The transgenic animals can be homozygous or heterozygous for the transgene. Mice are a preferred subject animal.

Construction of Transgenic Animals

Methods of making transgenic animals, e.g., mice, are known in the art. One approach is described below.

Injection/Implantation of Embryos

Procedures for embryo manipulation and microinjection are described in, for example, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., 1986, the contents of which are incorporated herein by reference). Mouse zygotes can be collected from six week old females that have been super ovulated with pregnant mares serum (PMS) followed 48 hours later with human chorionic gonadotropin. Primed females are placed with males and checked for vaginal plugs on the following morning. Pseudo pregnant females are selected for estrus, placed with proved sterile vasectomized males and used as recipients. Zygotes are collected and cumulus cells removed. Furthermore, blastocytes can be harvested. Pronuclear embryos are recovered from female mice mated to males. Females are treated with pregnant mare serum, PMS, to induce follicular growth and human chorionic gonadotropin, hCG, to induce ovulation. Embryos are recovered in a Dulbecco's modified phosphate buffered saline (DPBS) and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum.

Microinjection of a transgenic construct can be performed using standard micro manipulators attached to a microscope. For instance, embryos are typically held in 100 microliter drops of DPBS under oil while being microinjected. DNA solution is microinjected into the male pronucleus. Successful injection is monitored by swelling of the pronucleus. Recombinant ES cells can be injected into blastocytes, using similar techniques. Immediately after injection embryos are transferred to recipient females, e.g. mature mice mated to vasectomized male mice. In a general protocol, recipient females are anesthetized, paralumbar incisions are made to expose the oviducts, and the embryos are transformed into the ampullary region of the oviducts. The body wall is sutured and the skin closed with wound clips.

Screening for the Presence of the Targeting Construct

Transgenic animals can be identified after birth by standard protocols. DNA from tail tissue can be screened for the presence of the targeting construct using southern blots and/or PCR. Offspring that appear to be mosaics are then crossed to each other if they are believed to carry the targeting construct in their germ line to generate homozygous transgenic animals. If it is unclear whether the offspring will have germ line transmission, they can be crossed with a parental or other strain and the offspring screened for heterozygosity. The heterozygotes are identified by southern blots and/or PCR amplification of the DNA.

The heterozygotes can then be crossed with each other to generate homozygous transgenic offspring. Homozygotes may be identified by southern blotting of equivalent amounts of genomic DNA from mice that are the product of this cross, as well as mice that are known heterozygotes and wild type mice. Probes to screen the southern blots can be designed as set forth above.

Other means of identifying and characterizing the transgenic offspring are known in the art. For example, northern blots can be used to probe the mRNA for the presence or absence of transcripts encoding the reporter gene. In addition, western blots can be used to assess the level of expression of the transgene in various tissues of these offspring by probing the western blot with an antibody against the protein encoded by the transgene, or an antibody against the marker gene product, where this gene is expressed. Finally, *in situ* analysis (such as fixing the cells and labeling with antibody) and/or FACS (fluorescence activated cell sorting) analysis of various cells from the offspring can be performed using suitable antibodies to look for the presence or absence of the transgene product.

Other Transgenic Animals

Other transgenic animals can be used in methods of the invention. Methods for the preparation of a variety of animals are known in the art. A protocol for the production of a transgenic pig can be found in White and Yannoutsos, *Current Topics in Complement Research: 64th* ***Forum in Immunology*****, pp. 88-94**; US Patent No. 5,523,226; US Patent No. 5,573,933; PCT Application WO93/25071; and PCT Application WO95/04744. A protocol for the production of a transgenic rat can be found in Bader and Ganten, *Clinical and Experimental Pharmacology and Physiology*, Supp. 3:S81-S87, 1996. A protocol for the production of a transgenic cow can be found in *Transgenic Animal Technology, A Handbook*, 1994, ed., Carl A. Pinkert, Academic Press, Inc. A protocol for the production of a transgenic sheep can be found in

Transgenic Animal Technology, A Handbook, 1994, ed., Carl A. Pinkert, Academic Press, Inc. All patents and references are incorporated herein by reference.

Reporter Genes

The methods of the invention are based, at least in part, on coupling reporter genes to promoters of genes involved in skin metabolism. The reporter gene can be any gene which encodes a detectable product, preferably one which can be detected with relative ease, e.g., a gene product which is fluorescent, or which catalyzes a reaction which can be determined by formation of a colored product. For example, the reporter gene can encode an enzyme, e.g., an enzyme which produces a detectable product, e.g., a colored or a luminescent product. Reporter genes are known in the art and include a beta-galactosidase gene, a luciferase gene, a green fluorescent protein gene, an alkaline phosphatase gene, a horseradish peroxidase gene, or a chloramphenicol acetyl transferase gene. Reporter genes are described in, for example, Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

EXAMPLES

Example 1: Construction of the Transgene

To construct the transgene, an 839 bp fragment of the human versican promoter (nucleotides -559 to +280; the transcription start site being +1) was obtained from human genomic DNA using art known PCR-based techniques. The versican promoter fragment was inserted into the pNASS2β vector (Clontech) which was linearized by restriction with *Xho*I-*EcoR*I as shown in Figure 1. This vector contains the β-galactosidase reporter gene (lacZ) as well as a polyadenylation signal. This vector also includes an RNA splice donor and acceptor sequence to optimize the chance of high level of transgene expression. To obtain the linearized transgene DNA fragment for pronuclear injection, the vector was cleaved with an *EcoR*I and an *Xba*I enzyme. The linearized transgene DNA fragment has a length of 4732 bp.

Example 2: Generation of Transgenic Mice

The linearized transgene DNA fragment was injected into fertilized oocytes of DBA2 x C57BL6 (DBF1) mice (Charles River, Boston), and the eggs were implanted into pseudopregnant foster mothers. The offsprings (F0) were tested for chromosome integration of the human versican promoter fragment by southern blotting. Briefly, genomic DNA was isolated from the tails of 3-week old mice and digested with either

the *BamH*I or the *Pst*I restriction endonuclease. The DNA fragments were separated on a gel and then transferred on a nylon membrane. The membranes were hybridized with a 1.3 BP *BamH*I-*EcoR*V fragment of the human versican transgene which was used to generate the transgenic mice. Seven independent lines showed transgene insertion by Southern analysis. The copy number of the transgene varied from one to more than ten.

Example 3:* β*-galactosidase Histochemistry

Collected mouse embryos of varying stages (from E11.5d to E17.5d) of development were fixed with 0.5% glutaraldehyde in PBS for 30 minutes to 12 hours, depending on the embryo stage, transferred to 30% sucrose, and then frozen in OCT (Tissue Tek, CA) compound. 15μm-thick sections were prepared and loaded on slides. The sections were re-fixed in same fixative for 5 minutes and washed once with PBS, followed by a detergent wash solution for 10 minutes. Staining was performed in the reaction buffer containing 1 mg/ml X-gal (Sigma), 10 mM ferrocyanide, and 10 mM ferricyanide. Incubation was curried out for 3-6 hours at 37°C. After washing with PBS, sections were either mounted or counterstained with eosin. For adult tissue, mice were perfused with the same fixative and the desired organs were dissected and post-fixed, sucrose submerged, and then frozen with OCT compound. For skin tissue, 5 mm strips of back skin tissue block were fixed for 15 minutes to an hour and stained with X-gal, as described above for 3-6 hours. After washing, the skin tissue strips were embedded in paraffin and cut in 8 μm sections using a microtome.

***Example 4: In situ* hybridization**

Radioactive *in situ* hybridization with 3' end labeled oligonucleotide probes was performed using methods known in the art. The specificity of the *in situ* signal was tested by hybridizing some sections with labeled oligonucleotides in the presence of excess unlabelled oligonucleotide.

The expression pattern of the transgene was examined by *in situ* hybridization of transgenic embryonic sections (E13.5) using lacZ and endogenous mouse versican probes. Using either probe, expression was observed in the developing limb bud, kidney, brain, and cartilage. LacZ expression was also examined by β-galactosidase histochemistry in E13.5, E15.5, E17.5 embryos, through 7-day, 40-day newborns, and 4 month (adult) transgenic mice sections. Strong mesenchymal expression was observed in the kidney, brain, cartilage, and limb bud, as early as E13.5d. The level of expression remained constant until birth. In 7-day old mice, the β-galactosidase staining was

decreased compared to embryonic tissue and almost no expression was observed in adult tissue. In contrast to other tissue, dermal papilla (anagen hair cycle) from 30-day old mice exhibited intense β-galactosidase staining again and continued to express the same level of β-galactosidase during the second hair cycle.

In skin tissue, hair bud (future dermal papilla in hair follicle) expressed strong β-galactosidase activity at E15.5d and continued to do so until 7 days after birth (during the first hair cycle). Occasional β-galactosidase staining in dermal fibroblasts was also observed in E15.5d -newborn skin. However, β-galactosidase staining was decreased in 7 day-old skin tissue.

Example 5: **UV irradiation**

Irradiation with UVA was performed using a closely spaced array of five PUVA lamps. The energy output at 30 cm from array was measured with a UVA detector.

4-day old versican transgenic mice were irradiated on back skin with 30 J/cm2 UVA. Back skin tissue biopsy samples were processed 24 hours later for β-galactosidase staining and compared to untreated control skin from transgenic mice. β-galactosidase histochemistry showed increased β-galactosidase staining on the upper dermis of UVA irradiated skin compared to control.

Example 6: **VEGF-GFP Transgenic Mice**

To elucidate the regulatory mechanisms of human VEGF gene expression *in vivo,* transgenic mice have been prepared which contain a green fluorescent protein reporter construct driven by a 3kbp fragment of the promoter region of human VEGF (shown in Figure 2). This construct was confirmed to be functional *in vitro* by transfection assays in cultured human keratinocytes. Three independent transgenic lines for VEGF-GFP were obtained and confirmed by PCR analysis. To examine constitutive endothelial expression, and keratinocyte inducible VEGF expression in wound skin, 2 mm skin punches were made in the back skin and biopsy samples were collected after 48 hours. Tissues were immediately fixed with 4% paraformaldehyde and cryostat sections were examined by fluorescent and confocal microscopy. VEGF-GFP transgenic lines exhibited bright GFP fluorescence in neomicrovascular networks beneath the epidermis and upper dermis, and occasionally in the deeper dermis. Hair follicles were also positive in some tissue. In wound tissue, continuous GFP expression was observed at the wound edge of the epidermis, but no keratinocyte expression was seen in non-wounded areas, confirming the inducibility of VEGF expression in healing wounds.

Primary keratinocyte cultures prepared from the VEGF-GFP transgenic line also showed GFP fluorescence after 3 days in culture. Taken together these results indicate that GFP is a potentially useful tool to examine *in vitro* and *in vivo* VEGF expression. This work is described in more detail below.

Dynamic changes in the expression of green fluorescent protein driven by the human vascular endothelial growth factor promoter in transgenic mouse skin was analysed as follows..

Gene expression studies using transgenic mice most often utilize one of three classical reporter genes—lacZ (b-galactosidase) luciferase, or CAT (Chloramphenicol acetyl transferase). Detection of reporter gene activity usually requires the tissue to be removed from the animal for histochemical staining in the case of lacZ, or for complicated assays for luciferase and CAT, eliminating or at the least complicating the possibility of seeing a change in gene regulation in the living animal.

Recently an alternative fluorescent reporter molecule, green fluorescent protein (GFP), (Chalfee M, Tu Y, Euskirchen G, Ward WW and Prasher DC: Green fluorescent protein as a marker for gene expression. *Science* 263: 802-5, 1994) or modified GFP (EGFP), (Zhang G. Gurtu V and Kain SR: An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem Biophys Res Commun* 227: 707-11, 1996) has been developed. Because GFP is intrinsically fluorescent, the signal is visible without treatment (Misteli T and Spector DL: Applications of the green fluorescent protein in cell biology and biotechnology. *Nat Biotechnol* 15: 961-4, 1997). When GFP is induced in the epidermis, it is possible to observe changes in gene expression without sacrificing the animals using confocal laser microscopy. The promoter of human vascular endothelial growth factor (VEGF), which is an angiogenic factor that induces *in vivo* angiogenesis and vascular permeability in malignant tumor tissue is used here to express GFP as a reporter gene in transgenic mouse skin. The expression of VEGF in epidermal keratinocytes was shown to be up-regulated at the edge of a healing epidermal wound, and also up-regulated topical application of phorbolesters, such as TPA.

The expression and responsiveness of GTP in signaling changes in gene activity of human VEGF-GFP in transgenic mice is described below. The expression patterns are consistent with that of endogenous VEGF, and show that GFP-derived fluorescent can be localized and visualized using confocal microscopy on intact tissue without any treatment following excisions.

Transgene construct

Because PCR-based amplification of the GC-rish VEGF promoter is often difficult, a 5.0 kb fragment of VEGF genomic clone, which included the 5'-flanking DNA of human VEGF, was isolated from a human genomic library (Clontech, CA) using 500 bp cDNA fragment obtained by RT-PCR as a probe. The identified close was analyzed by restriction digestion, then a 2453 bp EcoRI-AgeI fragment (-2271 to +91) (Tischer E, Mitchell R, Harman T, Silva M, Gospodarowicz D, Fiddes JC and Abraham JA: The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266: 11947-54, 1991) was excised from the agarose gell. This promoter fragment was inserted into a GFP vector (pEGFP-1 cut EcoRI and XmaI). To maximize the efficiency of expression, 16s/19s splice donor and acceptor signals were also inserted between the promoter and GFP gene.

In vitro expression of GFP vector.

The resultant transgene in the mammalian expression vector was first transfected into cultured human keratinocytes to confirm that the selected gene region promoted VEGF expression. Primary human keratinocytes were transfected with either the BEGF-GFP vector or a CMV promoter-driven GFP control vector (pEGFP-N1; Clontech). A porybrene and DMSO method was used for all transfections.

Generation of Transgenic Mice

The linearized fragment for pronuclear injection was excised with EcoRI and AflII, which also included a polyadenylation signal sequence. This transgene fragment was then injected into fertilized oocytes of DBA2xC57Bl6 (DBF1) mice (Charles River Laboratories, Boston, MA), and the eggs were implanted into pseudo pregnant foster mothers. The offspring (F0) were tested for chromosomal integration of the transgene by genome PCR. All experiments were done with F1 or F2 offspring mice.

For confocal microscopic observation of intact skin tissue, VEGF-GFP transgenic mice were generated in the *hairless* genetic background by breeding transgenic founder with SKH-1 *hairless* mice (Charles River Laboratories). Thus, male hemizygous transgenic mice (v/-HH) were mated with female *hairless* mice (-/-hh) to obtain the F2 generation of VEGF-GFP hairless mice (v/-hh), which could be easily selected by PCR detection and their *hairless* phenotype.

Tissue preparation for GFP detection and epimicroscopic observation solutions washed out or diffused GFP-derived fluorescence from the site of GFP expression. Ultimately, a freshly dissected, unfixed tissue block, was directly cut into 12 μm thick frozen sections using a cryostat. Epifluorescent microscopic observation was performed immediately after sectioning, without mounting medium or extended slide storage, using FITC/TRITC double excitation and an emission filter Chroma, *VT) to distinguish the GFP-derived fluorescent signal from tissue autofluorescence.

Wounding and TPA treatment procedure

Skin wounds were produced with a 2 mm biopsy punch on the shaved back skin of young adult transgenic mice aged 4-6 weeks old. After 48 hours, normal and wounded tissues were collected and immediately frozen on dry ice.

For induction of VEGF mRNA by TPA, a single dose of 5 μg TPA in acetone was topically applied to back skin of transgenic mice and skin biopsy samples were collected after 12 hours.

All animal procedures had the approval of the MGH Animal Care and Use Committee.

Anti-VEGF and anti-GFP immunohistochemistry

Cryostate sections of transgenic mice skin were fixed with 4% paraformaldehyde in PBS (pH 7.2) at room temperature for 5 min. Immunohistochemical staining was performed on slide mount sections using the ABC method as described by the manufacturer (Vector Labs. UK), and visualized by peroxidase reaction using DAB as a color substrate. Anti-GFP antiserum was purchased from Clontech.

Confocal microscopy

For confocal microscopy observations, a coverslip was placed directly on the tissue. Sections or intact skin samples were analyzed using Leica TCS NT4D confocal microscope system with band pass filter 530/30nm, detecting emission at wavelengths between 515-545 nm. To visualize nuclei, sections were counterstained with 5μg/ml of 7-aminoactinomycin D (SIGMA, USA) solution for 20 min at ambient temperature, washed with PBS, and mounted with fluoromount-G (Southern Biotechnology, AL).

VEGF promoter driven GFP expression in human keratinocytes

As the region of functional promoter activity for endogenous VEGF was not well characterized, 2.5 kb of 5'-flanking DNA of human VEGF was first inserted in a GFP mammalian vector and monitored in transfected primary human keratinocyte cultures. Control cultures were transfected with a CMV promoter-driven GFP vector, VEGF-GFP fluorescence was first visible at 48 hours after transfection, and only in a small percentage of the cells. CMV-GFP fluorescence was visible within 24 hours, and nearly all the cells were positive. The VEGF-GFP signal was similar in intensity to that of CMV-GFP control confirming that this construct is a functional in *in vitro* culture system. (Fluorescence in cultured keratinocytes transfected with human cytomegalovirus (CMV) immediate early promoter driven GFP as a positive control, and with VEGF-GFP, 48 hours after transfection were observed.)

Expression of GFP in transgenic mice

In 30 founder mice, four lines positive for transgene chromosomal insertion were detected by PCR analysis. Three of these lines showed detectable GFP-derived fluorescent signal in the epidermis as assessed by epifluorescence microscopy of tail tissue, compared with negative wild type mice. GFP expression in new born transgenic mouse (Fluorescence was determined in fresh, unfixed tissue from the tail from a VEGF-GFP transgenic mouse and a similar tissue from a.negative wild-type litter mate. Fluorescence was also determined in tissues taken from new born VEGF-GFP transgenic mice: including lung (fluorescence seem in alveoli); lateral ventricle of the brain. (fluorescence was observed in lateral epithelium); and vertebral cartilage; (heart epithelium). The expression pattern of VEGF promoter was evaluated in F1 newborn pups of these three lines. The lung, kidney, and brain, previously been shown to express VEGF by in situ hybridization studies were chosen for examined for GFP fluorescence. As expected, GFP expression in lung alveoli and in the lateral ventricle wall in the brain was observed. It was difficult to detect a GFP signal in the flomerulus of kidney. GFP fluorescence was also detectable in the chondrocytes of developing cartilage tissue (e.g. vertebra cartilage).

GFP is expressed at the edge of healing wounds

Since non-stimulated, normal epidermal keratinocytes showed only weak GFP fluorescence, we tested the inducibility of VEGF-driven GFP during wound healing. 48 hrs after removal of a skin biopsy, the healing wound epidermis beneath the scab showed a very strong and distinct fluorescent signal compared with adjacent unwounded epidermis. This fluorescent signal correlated with VEGF/GFP expression detected by

anti-GFP and anti-VEGF immunohistochemistry in adjacent sections. GFP expression induced by skin wound healing. (VEGF-GFP transgenic mice were wounded by punch biopsy. Tissue from the wound was taken for evaluation by fluorescence microscopy. (a) In healing wound edge 48 hours after biopsy strong fluorescence was observed in the epithelium underlying the scab. (b) An adjacent section was reacted with anti-GFP antibodies. (c) An adjacent section was immunostained with antimouse VEGF antibodies. A control section was treated with normal goat serum. Non-specific peroxidase staining observed in the scabs as also seen in b and c.)

Induction of GFP by TPA treatment

Induction of epidermal GFP by phorbolester application was also examined. Accumulation of fluorescent signal was observed 12 hours after TPA was applied to transgenic skin, whereas acetone alone showed only basal levels of GFP expression. This signal seemed to localize to the basoplateral surface of the epithelium rather than in either the keratinocyt cytosol or nucleus. Anti-GFP immunostaining produced a signal in the basal cell cytosol, indicating that GFP originated in basal cells, and at least part of the GFP protein remained within the cell.

Direct detection of GFP by confocal microscopy

In order to localize the GFP fluorescence signal more precisely, fresh cryostat sections were fixed and incubated with 7-AAD for nuclear counter staining and examined under laser confocal microscope. As expected, most diffusable GFP was lost during this procedure, although wound healing-induced-epidermal expression remained unaffected. Occasional fluorescence was detectable in the unwounded epidermis, where the fluorescence appeared to localize outside basal cells as we observed. Even after fixation and intense washing, GFP-derived fluorescence in the outer root sheath of hair follicle remained unchanged. At higher magnification of the hair follicles, it can be seen that the fluorescent GFP signal completely co-localized with a nuclear maker, indicating that hair follicle GFP remains stably within the nucleus. This was also the case for the chonodrocytes in the vertebrae cartilage.

Direct detection of GFP in the epidermis of intact skin by confocal microscopy

To test the possibility that GFP might be detected in the skin of a living GFP transgenic mouse, dissected *hairless* skin was directly examined by confocal imaging. Horizontal optical sections of the living epidermis revealed bright fluorescence. The equivalent cryostate section showed that this fluorescence is derived from the basal cells.

Higher magnification revealed that GFP was localized in the extracellular space around the keratinocytes. X-Z confocal imaging confirmed that this signal was within the basal layer of the epidermis. Detection of GFP expression in intact skin by confocal microscopy. Fress unfixed transgenic mouse skin was biopsied, and immediately evaluated by scanning laser confocal microscopy. Horizontal optical sections of the epidermis show fluorescence surrounding individual epithelial cells. Cryostate sections of an equivalent skin region counterstained with 7-AAD to indicate nuclei, showed fluorescence at the dermal-epidermal junction. The confocal X-Z image indicates the fluorescence near the basal keratinocytes.

This work shows that the expression pattern of GFP driven by a human VEGF promotor is spatially and temporally equivalent to that seen with mRNA detection.

Pups from breedings of transgenic mice expressing GFP in the skin epidermis were easily selected by simple epifluorescent microscopy, without the more tedious procedures of southern blotting or genomic PCR.

There was initial difficult in detecting GFP-derived fluorescence in sections, even though fresh untreated tissue blocks emitted bright fluorescence. After testing a variety of procedures, it was found that aqueous solution treatment immediately washed out the GFP flourescence. This was partially overcome by fixing the tissue block before cryostate sectioning, though fluorescence from some sites, such as that initially present in epidermal keratinocytes, was still lost or its staining pattern was altered. Fixation induced fluorescence indistinguishable from that produced by GFP in the skin microvasculature. Fluorescence at this site was not seen in unfixed tissue, and VEGF expression has not been reported in the microvasculature in studies using *in situ* hybridization methods. Thus, the most efficient method to preserve the original GFP signal is to immediately cryostat section freshly dissected skin without OCT compound, and then promptly examine it by fluorescence microscopy. This unexpected difficulty may explain why, until now, there have been so few reports of successful GFP transgenic mice.

Prominent VEGF expression has been reported in lung, and in the lateral ventricle of the brain by *in situ* hybridization. Evaluation of the VRGF-GFP mice tissues confirm these findings. Failure of the kidney glomeruli to fluoresce may be due to a lack of tissue specific response element to 2.2 kb of 5'-flanking region of VEGF DNA used here for generating these transgenic mice, or to a signal below the detection threshold.

In skin from a 1 week old pup, normal epidermal keratinocyte showed basal levels of GFP expression, while bright fluorescence was observed in the outer root sheath of hair follicle. The skin of older mice did not show equivalent GFP expression in hair follicles, therefore, VEGF expression in the follicles may be hair cycle-dependent. Fluorescence in hair follicle and in chondrocytes, as seen in the vertebra and previously reported was particularly impressive. These signals were even stronger than those observed in lung or brain. Laser confocal observations revealed that GFP at these locations is confined within the nuclei. This nuclear localization may intensify the apparent signal since nuclear localization is likely to reduce the diffusion of the fluorescent product during tissue processing. The reason for localization to the nucleas by some tissues and not by others remains unexplained, but suggests that inclusion of a nuclear localization conseneus sequence with in the transgene might be useful.

The inducibility of GFP protein during wound healing model and after TPA treatment is shown herein, confirming that the reporter GFP expression pattern is highly VEGF promoter-dependent. Also, these data eliminated concerns about the possible delay in fluorescent signal production due to slow chromophore formation as reported in Drosophila embryos. (Davis I, Girdham CH, O'Farrell PH "A nuclear GFP that marks nuclei in living Drosophila embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence" *Dev. Biol.* 170:726-9 (1995)). In anticipation of this difficulty the red-shifted variant of GFP was selected for these studies, and the correct folding of the chromophore appears to occur in skin as well as in more internal tissues. There was also concern that GFP might have a prolonged half life in skin interfering with the ability to use the reporter to monitor long-term gene expression. The disappearance of fluorescence in hair follicles of older transgenic mouse skin indicates that GFP protein has a reasonable half life.

Laser confocal microscopic evaluation of intact skin for detection of the GFP signal indicates that GFP is readily detected within the epidermis. These results suggest the potential use of GFP transgenic mice for non-invasive monitoring of long-term gene expression *in vivo*. The technology for microscopic evaluation of living mouse skin is already in use. When combined with use of a transgenic GFP reporter gene, the system has a great advantage over conventional transgenic reporter gene animals methods because it reduces the number of animals used, utilizes a simple monitoring system, and allows long term monitoring of changes in gene expression on the same individual. Our successful *in vivo* expression of GFP in transgenic mouse skin should facilitate the understanding of VEGF gene expression in skin, by providing a useful monitoring and

screening tool. Its use would be particularly attractive when bred to a mouse with a phenotype thought to involve VEGF expression. This model would also be advantageous in research focused on skin homeostasis during the aging process.

Example 7: Developmental and Age-Related Changes of Human Versican Promoter Activity in Transgenic Mice Skin and Hair Dermal Papilla

To investigate further for temporal-spacious expression pattern for human versican, particularly in skin tissue, transgenic mice were generated containing a lacZ reporter construct driven by the functional promoter for the core protein of human versican and tissue sections from resultant transgenic mice ranged from embryos, newborn to adult stage were examined by b-galactosidase histochemistry.

Construction of transgene

The 839 bp fragment of functional human versican promoter (from -559 to +280 according to the transcription start site as +1) including exon 1; (Naso MF, Zimmermann DR and Iozzo RV, "Characterization of the complete genomic structure of the human versican gene and functional analysis of its promoter", *J. Biol. Chem.* 269:32999-3008 (1994)) was obtained from human genomic DNA by PCR based technique and correct sequences were confirmed by direct sequencing. pNASS2β vector (Clontech) was used as a source of β-galactosidase reporter gene (lacZ) with polyadenylation signal. This vector also included RNA splice donor and acceptor sequence to optimize the chance of high level of transgene expression. Versican promoter fragment was inserted in front of splice donor/acceptor sequence using XhoI - EcoRI restriction site. Linearised transgene DNA for pronuclear injection was obtained as EcoRI-Xbal 4732bp fragment.

Generation of Transgenic Mice

The linearized construct was injected into fertilized oocytes of DBA2xC57BL6(DBF1) mice (Charles River, Boston), and the eggs were implanted into pseudo pregnant foster mothers. The offspring(F0) were tested for the chromosome integration of the human versican promoter construct by Southern hybridization. Thus, genomic DNA was isolated from the tails of the mice at 3 weeks old and digested either BamHI or PstI restriction endonuclease. The fractionated DNA fragment on the nylon membranes were hybridized with 1.3kbp BamHI-EcoRV fragment of human versican transgene were used to generate transgenic mice. Seven independent lines which show

transgene insertion by Southern analysis with different copy number from single insertion to more than ten.

b-galactosidase histochemistry

Collected mouse embryos of early to mid stages (from E11.5d to E15.5d) of development were fixed with 0.5% glutaraldehyde in PBS for 30 min to 12 hrs depends on the stage and transferred to 30% sucrose, then frozen in OCT compound. Sections were cut 15um thickness and loaded on slides. Sections were re-fixed in same fixative for 5 min and was once with PBS, followed by detergent wash solution for 10 min. Staining reaction was done in the reaction buffer containing 1mg/ml X-gal (Sigma), 10mM ferrocyanide, and 10mM ferricyanide. Incubation was curried out for 3-6 hrs at 37°C. After washing with PBS, sections were either mounted or counterstained with eosin. For adult tissue, mice were perfused with same fixative and desired organs were dissected and post-fixed, sucrose submerged, then frozen with OCT compound. In order to preserve better morphology some early to mid embryo (from E11.5d to E15.5d) were processed for paraffin section. For skin tissue, 5mm strip of back skin tissue block were fixed for 15 min-1 hr and proceed X-gal staining for 3-6 hrs. After wash, blocks were processed for paraffin embedding and cut 8 um section with microtome.

In situ *hybridization*

The embryo tissues for in situ hybridization were fixed freshly prepared 4% paraformaldehyde in DEPC-treated PBS, dehydrated, processed through a standard paraffin embedding protocol under RNase-free condition. Digoxigenin labeled non-radioactive in situ hybridization was performed on 8 um paraffin sections of E13.5d embryo as described previously (Kishimoto J, Cox H, Deverne EB, and Emson PC "Cellular Localization of Putative Odorant Receptor Messenger RNAs in Olfactory and Chemosensory Neurons - A Non Radioactive Insitu Hybridization Study" *Molecular Brain Research* 23:33-39 (1994)). CDNA for lacZ and mouse versican antisense probe were prepared by PCR and sub-cloned into pBluescriptII(stratagene). Digoxigenin labeled RNA probes were prepared using a RNA labeling kit (Boehringer) according to the manufacturers' instruction. Paraffin sections on slides were quickly dewaxed in xylene, dehydrated through 100%, 90%, 70% ethanol, washed with 0.1M PBS (pH7.5) and then treated with 0.2N hydrochloric acid for 10 minutes. Sections were treated with 0.02% pepsin (in 0.2N HCl) for 30 minutes at 37°C, following which the enzyme was deactivated by 4% paraformaldehyde. After rinsing in 0.2% glycine solution (in PBS)

three times, slides were acetylated in 0.25% acetic anhydride in 0.1M triethanolamine / 0.9% NaCl for 10 minutes at room temperature and partially dehydrated through 70, 80, 90% ethanol and briefly dried. Approximately 0.1μg/ml digoxigenin labeled RNA probes (denatured by boiling prior to use) were added in hybridization buffer (50% deionized formamide, 4XSSC, 10% dextran sulphate, 1X Denhardt's solution) and slides were incubated at 55°C under coverslips for 16-18 hours. The coverslips were carefully removed and the sections were washed in 2X SSC/0.1% SDS for 30 minutes at room temperature, sequentially washed twice in 0.1X SSC / 0.1% SDS at 60°C for 30 minutes. Immunohistochemical procedures to visualize the hybridized probes used alkaline phosphatase conjugated anti-manufactures' instructions. The colorimetric reaction was started by adding nitroblue tetrazolium (Boehringer) and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer) and incubated for 6-24 hours at room temperature. Slides were mounted with glycerin jelly and were kept at 4°C.

Radioactive in situ hybridization with 3' end labeled oligo nucleotide probes was carried out as described previously (Kishimoto J. Keverne EB, Hardwick J, Emson PC, "Localization of nitric oxide synthase in the mouse olfactory and vomeronasal system: a histochemical, immunological and in situ hybridization study" *European J. of Neuroscience* 5:1684-1694 (1993)). Sequences of mouse versican antisense oligonucleotide probes were 5'-CCGTTCTGGGCCACCAAGACAGTCGTCTCC-3', 5'-TGACCGCCCCGATATCCAAACAAGCCTGTT-3' (SEQ ID NO: ___), 5'-TCCGACAGCCAGCCGTAATCGCATTGGTCA-3' (SEQ ID NO: ___). Some sections were hybridized with labeled oligonucleotides in the presence of excess unlabelled same oligonucleotides to check the specificity of the *in situ* signal.

Cell culture

For primary fibroblast culture from transgenic mice skin, newborn transgenic mice skin were pealed off and incubated in 0.25% trypsin 18 hrs. Discarded epidermal sheet and dermis were further digested by collagenase for 1 hr. Cells were spread out to the MEDM medium (GIBCO)+ 10% FCS. cells were passaged in every 3 days after trypsin treatment. For b-galactosidase histochemistry cultured fibroblast cells were fixed with 0;.2% glutaraldehyde in PBS for 2 min., followed by detergent wash solution for 5 min. and staining reaction was performed for 18 hrs. at 30°C. After reaction was stopped cells were proceeded for counter-nuclear staining with 5μg/ml of 7-aminoactinomycin D (SIGMA, USA) solution for 20 min. at R.T. then, washed PBS, mount with fluoromount-G (Southern Biotechnology, AL).

Generation of hairless versican-lacZ transgenic mice

To eliminate hair follicle derived lacZ expression in order to examine age-related change of versican promoter activity, hairless genetic background of versican-lacZ transgenic mice were obtained by breeding with SKH-1 hairless mice resultant founder with SKH-1 (Charles River Laboratories, Boston). Thus, male hemizygous transgenic mice (v/-HH) were mated with female hairless mice(-/-hh), and selected transgene positive pups (v/-Hh) by genomic PCR were then mated back to a hairless again to obtain some F2 generation of versican-lacZ hairless mice(v/-hh) which can be easily selected by combination of PCR detection and hairless appearance.

Generation of versican-lacZ transgenic mice

Of a total of 27 founder mice six positive lines were detected by both PCR and southern blotting analysis for the transgene insertion. The copy number of transgene was 1 to more than 20 copies per cell. These were further screened for lacZ staining by using X-gal histochemical reaction on tail skin. The intensity of lacZ staining in tail did not correlated with the copy number of transgene as both two lines. A4681(1-2 copies) and A4688(over 20 copies) exhibited similar strong lacZ staining in tail hairs with A4679 which had also over 20 copies, showed only light staining in tail. The transgenic line A4681 was chosen for further study as the staining of tail skin was most intense and had a good breeding behavior. All further analysis was performed on F1-F3 offspring derived by mating with DBA/2 strain.

Distribution of lacZ expression in embryonic tissue

Intense lacZ staining was observed in developing fore and hind limb (E13.5) within an area of mesenchymal condensation and also in ectoderm. This ectodermal expression was restricted in the tip of limb. In situ hybridization with radio-labeled antisense oligoes showed similar endogenous mouse versican mRNA expression in condenses mesenchyme in limb bud.

In E15.5 embryo strong lacZ staining was also observed in kidney glomeruli mesenchyme in olfactory epithelium, mesenchyme and muscles in tongue, and submandibular gland. Perichondrocyte surrounding cartilage, blood vessel, developing edge of hind and fore brain, smooth muscle were also positive.

The expected expression pattern other than limb was confirmed by digoxigenin non-radioactive in situ hybridization with lacZ and endogenous mouse versican probes

on transgenic embryonic section (E13.5) as well as control sense probes. Both mRNA expression was observed in; kidney, olfactory epithelium, humerus cartilage, and in vertebrae cartilage, confirming reasonable expression pattern and timing of expression of this transgenic mice.

Human versican activity in embryonic skin

LacZ expression was examined by X-gal histochemistry on developing skin from E13.5, E14.5, E15.5 and E17.5 embryo. At E13.5d basically no staining was observed in whole body of ectoderm except hind and fore limb and with a few occasion trace of lacZ staining was found in single mesenchymal cell which seems to be earliest stage of condensation. Hair germ of whisker has already exhibited lacZ staining at this stage. At E14.5 condensed mesenchymal cells attached beneath ectodermal placode (hair plug) were clearly lacZ positive and this was highly contrasted against complete negative surrounding scattered mesenchymal cells. Phase contrast photo showed individual b-gal positive condensed mesenchymal cell. The number of these positive site were 4 -5 per sagittal - mediaeval embryonic section of whole embryo tissue. At E15.5 these positive condenses mesenchyme under hair plug were getting more intense and increased in volume (i.e. the number of cell). At E17.5 the number and intensity of lacZ positive dermal papilla are dramatically increased, yet still highly restricted within the future dermal papilla. A few epidermal placode (i.e. epithelial keratinocytes) itself exhibited lacZ staining although the majority were still restricted in mesenchymal cells.

Hair cycle dependent human versican activity in transgenic mice skin

LacZ staining was examined on the skin section of transgenic mice from newborn, 7 days, 14 days, 28 days, 40 days, to 4 month old. Strong lacZ staining in hair dermal papilla cells in late embryonic skin were proceed into new born skin and continue during first anagen hair cycle. Strong staining was confined within the dermal papilla cells. Interestingly at age 7, mid to late anagen stage relatively strong staining was also observed in inner root sheath. However, in late anagen stage lacZ staining was again restricted only in dermal papilla cells. Also later in telogen hair no lacZ staining observes in club hair. In second hair cycle growing dermal papilla (second anagen hair cycle) exhibited again intense lacZ staining and after second anagen hair cycle lacZ activity in hair dermal papilla was decreased almost completely through long telogen hair cycle.

Human versican activity in cultures fibroblast

Primary dermal fibroblast culture derived from dermal fibroblast cells of new born transgenic line exhibited lacZ staining in only certain population of cells and center of hair clamp, obviously dermal papilla. Since all strongly stained cells were round shape these should be derived hair dermal papilla. After first passage, however, shape of positive cells were diversed and not confined round shape, including typical fibroblast-like cells with dendritic process in passage 2. These population of positive cells maintained after first passage to at least until six passages we cultured while certain number of cells were completely unstained through passages determined with counter-nuclear staining which clearly indicated total number of cells. Approximate number of positive cells were not exceed over 50%. Intensity of staining was gradually faded with passages.

Age-related change of human versican activity in transgenic mouse skin

Increasing reaction time for x-gal staining to 18 hrs revealed more staining in upper dermis other than hair dermal papilla in young to adult skin of the transgenic mouse. In newborn mouse skin under this long incubation condition, most of dermis including hair follicle (hairless mouse have normal hair only in first cycle) showed fairly detectable staining. Young mouse skin showed only isolated fibroblast derived diffused staining preferentially in upper dermis, and older mouse only exhibited faint staining, indicating age-related decrease of human versican promoter activity in transgenic mouse skin.

Example 8: Evaluation of Reporter gene expression in a live animal.

Method:

Confocal microscopy

To examine whole intact skin on living transgenic mouse with confocal microscope, hairless VEGF-GFP transgenic mice were anaesthetized with avertin by intraperitoneal injection. They were placed directly on the petri dish with dorsal position. TPA or acetone treatment was performed on the back skin same as described above marking treated area with an ink to ease the orientation of the skin. Sections or intact skin of the mice were analyzed using Leica DM IRBE inverted microscope and Leica TCS NT4D confocal microscope system with band pass filter 530/30 nm, detecting emission at wavelengths between 515-545 nm.

Direct detection of GFP in the epidermis of intact skin of living hairless transgenic mice by confocal microscopy

To test whether the induction of GFP is directly detected in the skin of a living VEGF-GFP transgenic mouse, acetone or TPA applied skin of the transgenic *hairless* mouse (same individual) was directly examined by confocal imaging under anaesthetized condition. Horizontal optical sections through the normal living epidermis revealed detectable fluorescence and the skin treated with TPA after 12hrs showed the increase of fluorescence positive area. Non-transgenic wild type litter mate showed no detectable specific fluorescence. Higher magnification of the TPA treated skin revealed that GFP was localized in the extracellular space around the keratinocytes. X-Z confocal imaging confirmed that this signal was within the basal layer of the epidermis.

Laser confocal microscopic evaluation of an intact skin of living transgenic mouse for detection of the GFP signal indicates that GFP is readily detected within the epidermis. These results suggest the potential use of GFP transgenic mice for non-invasive monitoring of long-term gene expression *in vivo*. When the transgenic GFP reporter gene is combined with the technology for microscopic evaluation such as confocal microscope, the system has a great advantage over conventional transgenic reporter gene animals methods because it reduces the number of animals used, utilizes a simple monitoring system, and allows long term monitoring of changes in gene expression on the same individual.

Successful *in vivo* expression of GFP in transgenic mouse skin provides for the understanding of the regulation of VEGF gene expression, such as in inflammatory and neoplastic skin diseases, by providing a useful monitoring and screening tool. It is particularly attractive when bred to a mouse with a phenotype which involves VEGF expression. This novel experimentation model will allow in vivo studies on the regulation of VEGF gene. This GFP reporter system on transgenic animal would also be advantageous in research focused on skin homeostasis during the aging process.

Example 9: human MMP9 transgenic mice

MMP9 (gelatinase B, 92-kDa type IV collagenase) is one of the member of matrix metalloproteinases (MMPs) capable to degrade extracellular matrix (ECM) components. This enzyme (MMP9) is known to degrade type IV and V collagens, gelatin and elastin. It is also shown to be induced by UVB which causes photoaging. (Fisher, G.J., *Nature* **379**, 335, 1996). 5'flanking region of this gene from -670 sequence

position to +1 transcription start site is sufficient to drive expression of a reporter gene (CAT) in HT1080 cells. (Sato, H., *Oncogene* **8**, 395, 1993).

Construct of the Transgene

To construct the transgene, an 733 bp fragment of the human MMP9 promoter (nucleotides -714 to +19; the transcription start site being +1) was obtained from human genomic DNA using art known PCR-based techniques. Two different reporter genes, beta-galactosidase reporter gene (lacZ) and green fluorescent protein (GFP) gene, were employed for construction.

For the construction with beta-galactosidase reporter gene (lacZ), the MMP9 promoter fragment was inserted into an modified pNASSbeta vector (Clontech), which is inserted neutrophil elastase promoter and has Bgl II site, linearized by restriction with Bgl II-Xho I. To obtain transgene DNA fragment for pronuclear injection, the vector was cleaved with Bgl II and Hind III. The length of the linearized transgene DNA was 4630bp.

For the construction with green fluorescent protein (GFP) gene, the same MMP9 promoter fragment was inserted into the pEGFP-1-SV, which was modified vector to have splice donor/acceptor based on pEGFP-1 vector (Clontech), linearized by restriction with Hind III-Kpn I. To obtain transgene DNA fragment for pronuclear injection, the vector was cleaved with Hind III and Afl II. The length of the linearized transgene DNA was 1928bp.

Generation of transgenic mice

The lenearized transgene DNA fragment was injected into fertilized oocytes of hairless mice (SKH1; Charles River, Boston). Those eggs were implanted into pseudo pregnant foster mothers. The offsprings (F0) were tested for chromosome integration of lacZ or GFP fragment by southern blotting. For the detection of lacZ transgene, the genomic DNA extracted from the tail was digested with Nci I and separated on a gel, then transferred on a nylon membrane. The Nci I digested transgene DNA fragment was used for the probe. Two independent lines showed a transgene insertion.

For the detection of GFP transgene, the tail genomic DNA was digested with Hinc II Two independent lines showed a transgene insertion.

beta-galactosidase histochemistry

F1 mice obtained from founders (F0) were used for beta-galactosidase histochemistry. Bones of 2-wk-old mice hindlimb were fixed with 0.5% glutaraldehyde in PBS for 30 minutes. After three times washing with PBS and once with detergent wash, the tissue was incubated with reaction buffer containing 1 mg/ml X-gal (Sigma),

10 mM ferrocyanide and 10 mM ferricyanide at 37°C. After 2 hours incubation, blue staining was observed in a F1 mouse which has transgene, while no staining was observed in the sibling without transgene. These are consistent with a report in which MMP9 was shown to express at bone of 2-wk-old mouse limb by in situ hybridization (Reponen, P., *Journal of Cell Biology* **124**, 1091, 1994).

Example 10: human Neutrophil Elastase transgenic mice

Neutrophil elastase is one of the powerful serine proteinase capable attacking a broad range of proteins. Hairless mice deficient in this enzyme do not suffer from elastosis, which is one of signs of photoaging caused by UVB irradiation. (Starcher, B., *Connective Tissue Research*, **31**, 133, 1995). Therefore, neutrophil elastase is believed to play an important roll in photoaging.

Construction of the Transgene

To construct the transgene, an 1299 bp fragment of the human neutrophil elastase promoter (nucleotides -1280 to +19; the transcription start site being +1) was obtained from human genomic DNA using art known PCR-based techniques. This neutrophil elastase promoter fragment was inserted into the pNASSbeta vector (Clontech) linearized by restriction with Eco RI - Xho I. To obtain transgene DNA fragment for pronuclear injection, the vector was cleaved with EcoR I and Xba I. The length of the linearized transgene DNA was 5171bp.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS:

1. A method of evaluating a treatment for its effect on skin, comprising:

providing a transgenic animal having a reporter gene coupled to a skin metabolism-related promoter;

administering said treatment to said transgenic animal; and

evaluating expression of said reporter gene,

thereby evaluating said treatment for its effect on skin.

2. The method of claim 1, wherein said treatment includes a compound which d is administered by applying said compound to the skin of said transgenic animal

3. The method of claim 1, wherein said transgenic animal is selected from the group consisting of a transgenic mini-pig, a transgenic guinea-pig, a transgenic rat, and a transgenic mouse.

4. The method of claim 1, wherein said skin metabolism-related promoter is a human skin metabolism-related promoter.

5. The method of claim 1, wherein said skin metabolism-related promoter is selected from the group consisting of a promoter which controls the metabolism of an extracellular matrix protein, a versican promoter, a matrix metalloproteinase promoter, a vascular endothelial growth factor promoter, a hyaluronan synthase promoter, a MMP2 or MMP9, promoter, and a neutrophil elastase promoter.

6. The method of claim 1, wherein said reporter gene is selected from the group consisting of the genes which encode the beta-galactosidase, luciferase, green fluorescent protein, alkaline phosphatase, horseradish peroxidase, and chloramphenicol acetyl transferase.

7. The method of claim 1, further comprising a subsequent administration of said treatment.

8. The method of claim 1, further comprising comparing the expression of said reporter gene to a control value.

9. The method of claim 1, wherein said transgenic animal includes a reporter gene coupled to a second skin metabolism-related promoter, wherein the first skin metabolism-related promoter is different from the second skin metabolism-related promoter.

10. The method of claim 1, wherein the evaluation of the expression of the reporter gene step is repeated at least once during the life of the animal.

11. The method of claim 11, wherein the first and a subsequent repetition of the step can be separated by as much as 1, 10, 30, 60, 90, 180, 365, or 700 days.

12. The method of claim 11, wherein the first and a subsequent repetition are performed on a live animal.

13. A non-human transgenic animal having a reporter gene coupled to a skin-metabolism promoter.

14. The non-human transgenic animal of claim 16, wherein said promoter is selected from the group consisting of a versican promoter, a matrix metalloproteinase promoter, a vascular endothelial growth factor promoter, a hyaluronan synthase promoter, a MMP2 or MMP9, promoter, and a neutrophil elastase promoter.

15. A method of analyzing the expression of a transgene in a transgenic animal, comprising:

providing a live transgenic animal;

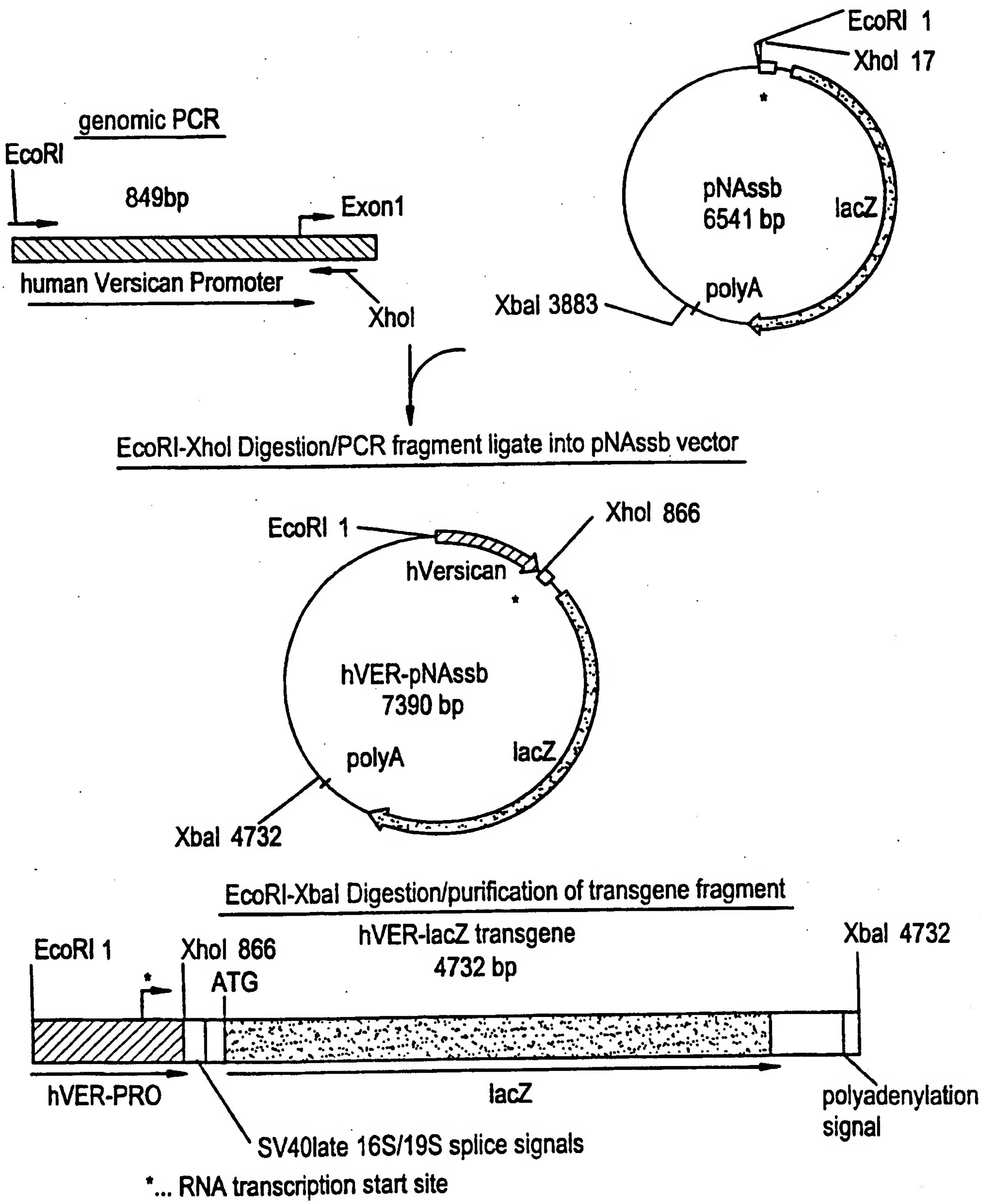
evaluating, in the live transgenic animal, the presence or absence of a reporter gene encoded by a transgenic sequence or under the control of a transgenic control element;

thereby analyzing the expression of a transgene.

16. The method of claim 15, wherein the reporter is GFP.

17. The method of claim 1, wherein the evaluation step is repeated at least once during the life of the animal.

FIG. 1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Exon1
human Versican Promoter
XhoI
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XhoI 17
pNAssb
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lacZ
polyA
XbaI 3883
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EcoRI 1
XhoI 866
hVersican
hVER-pNAssb
7390 bp
polyA
lacZ
XbaI 4732
EcoRI-XbaI Digestion/purification of transgene fragment
EcoRI 1
XhoI 866
ATG
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lacZ
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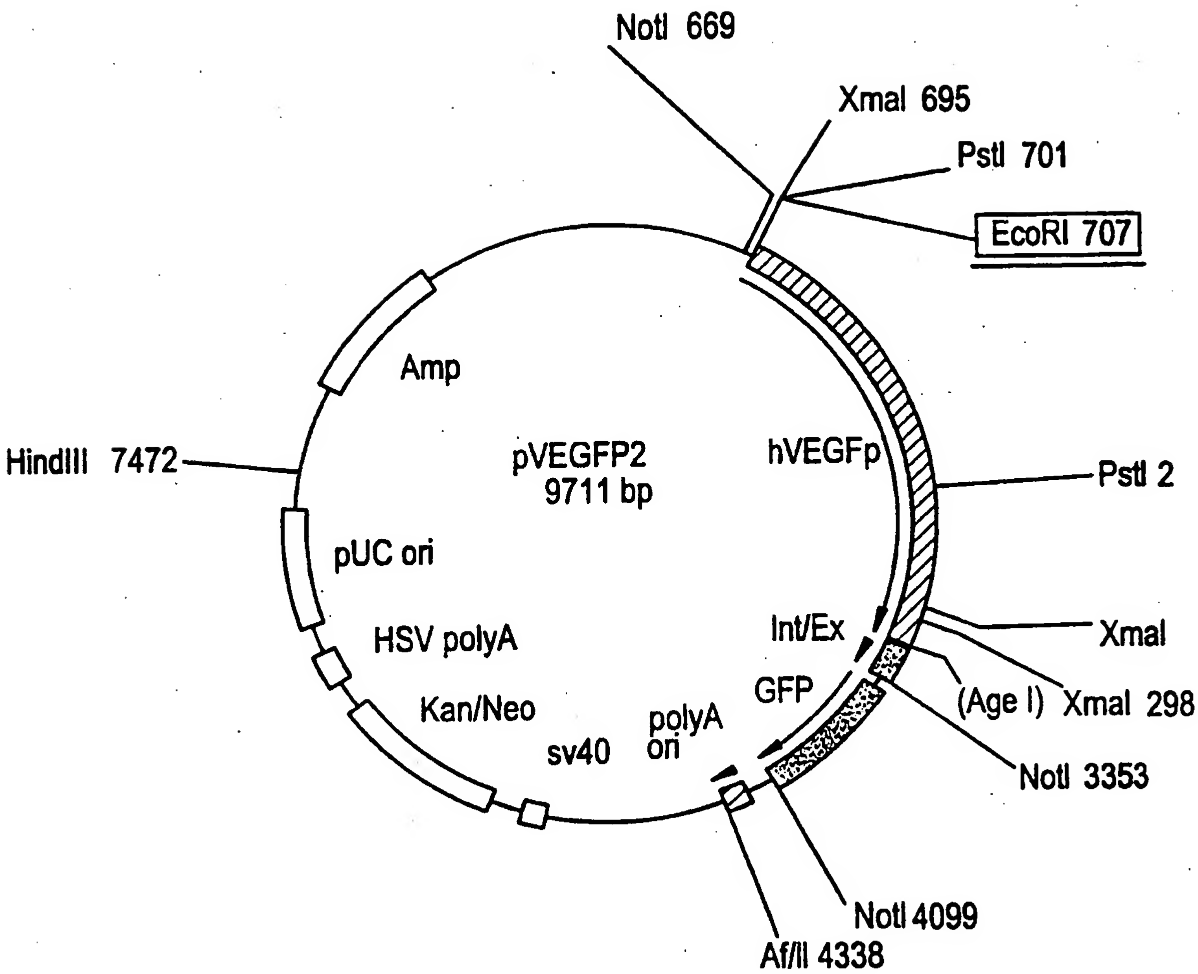
SV40late 16S/19S splice signals
*... RNA transcription start site

FIG. 2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hVEGFp
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pUC ori
HSV polyA
Int/Ex
XmaI
GFP
(Age I)
XmaI 298
Kan/Neo
polyA
ori
sv40
NotI 3353
NotI 4099
Af/II 4338

FIG. 3

TAGTTATTACTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCCCGAGGTCCTGAAGGAA
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CGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTC
CAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACC
GTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGG

FIG. 3 Cont.

TGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAA
GCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCT
GGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTAC
AGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCT
AAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATT
GAAAAAGGAAGAGTCCTGAGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGT
GGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGC
AACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCA
ATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTT
CCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTC
GGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGA
TCGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGT
TCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG
CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGA
CCTGTCCGGTGCCCTGAATGAACTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGA
CGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTA
TTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCC
ATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCAC
CAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGA
TGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGA
GCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATG
GTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTAT
CAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCG
CTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTT
GACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTG
CCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTC
CGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCT
AGGGGGAGGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGG
CAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGG
TCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATGGGGCCAATACGCCCGCGTTT
CTTCCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCG
GGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTC
ATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTT
AACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGA
GATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTG
GTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCG

FIG. 3 Cont.

CAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTA
GCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAG
TCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG
AACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACC
TACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC
GGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGG
TATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGT
CAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTT
TGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTA
CCGCCATGCAT

FIG. 4

GAATTCGCCTGGGTGAAAGTGAGTTCCCCGTTGGAGGCAACAGACGAGGAGAGGATGGAAG
GCCTGGCCCCCAAGAATGAGCCCTGAGGTTCAGGGAGCGGCTGGAGTGAGCCGGCCCCAGAT
CTCCCGAGGTCCTGAAGGAAGAGAGTAAAGCCATGTCTGCTGTTTTCTAGAGGCTGCTACTGT
CCCCTTTACTGCCCTGAAGATTCAGCCTGCGGAAGACAGGGGGTTGCCCCAGTGGAATTCCCC
AGCCTTGCCTAGCAGAGCCCATTCCTTCCGCCCCCAGATGAAGCAGGGAGAGGAAGCTGAGT
CAAAGAAGGCTGTCAGGGAGGGAAAAAGAGGACAGAGCCTGGAGTGTGGGGAGGGGTTTGG
GGAGGATATCTGACCTGGGAGGGGGTGTTGCAAAAGGCCAAGGATGGGCCAGGGGGATCAT
TAGTTTCAGAAAGAAGTCTCAGGGAGTCTTCCATCACTTTCCCTTGGCTGACCACTGGAGGCT
TTCAGACCAAGGGATGGGGGATCCCTCCAGCTTCATCCCCCTCCCTCCCTTTCATACAGTTCC
CACAAGCTCTGCAGTTTGCAAAACCCTACCCCTCCCCTGAGGGCCTGCGGTTTCCTGCGGGTC
TGGGGTCTTGCCTGACTTGGCAGTGGAGACTGCGGGCAGTGGAGAGAGGAGGAGGTGGTGTA
AGCCCTTTCTCATGCTGGTGCTGCCACACACACACACACACACACACACACACACACACACA
CACACCCTGACCCCTGAGTCAGCACTTGCCTGTCAAGGAGGGGTGGGGTCACAGGAGCGCCT
CCTTAAAGCCCCCACAACAGCAGCTGCAGTCAGACACCTCTGCCCTCACCCTCGAGGAACTG
AAAAACCAGAAAGTTAACTGGTAAGTTTAGTCTTTTTGTCTTTTATTTCAGGTCCCGGATCCG
GTGGTGGTGCAAATCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGCCTGTACG
GAAGTGTTACTTCTGCTCTAAAAGCTGCGGAATTGTACCCGCGGCCGCAATTCCCGGGGATCG
AAAGAGCCTGCTAAAGCAAAAAAGAAGTCACCATGTCGTTTACTTTGACCAACAAGAACGTG
ATTTTCGTTGCCGGTCTGGGAGGCATTGGTCTGGACACCAGCAAGGAGCTGCTCAAGCGCGA
TCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC
AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCA
ACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGC
CGGAAAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTGGC
AGATGCACGGTTACGATGCGCCCATCTACACCAACGTAACCTATCCCATTACGGTCAATCCGC
CGTTTGTTCCCACGGAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCT
GGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGT
GCAACGGGCGCTGGGTCGGTTACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGC
GCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGTTGGAGTGACGGCAG
TTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCA
TAAACCGACTACACAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTCAGCCG
CGCTGTACTGGAGGCTGAAGTTCAGATGTGCGGCGAGTTGCGTGACTACCTACGGGTAACAG
TTTCTTTATGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATT
ATCGATGAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCAAAACCCGAAA
CTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCGGTGGTTGAACTGCACACCGCCGACGG
CACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGTC
TGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCATCATCCTC

FIG. 4 Cont.

TGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAAC
AACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGCGAC
CGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCAATGAA
TCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAGCGAACGCGTAACGCGAATGGTGC
AGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGC
GCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTGCAGTAT
GAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGGA
TGAAGACCAGCCCTTCCCGGCTGTGCCGAAATGGTCCATCAAAAAATGGCTTTCGCTACCTGG
AGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGTCTTGGCGGTTT
CGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCGTTTACAGGGCGGCTTCGTCTGGGACTG
GGTGGATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCGTGGTCGGCTTACGGCGGTG
ATTTTGGCGATACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCA
CGCCGCATCCAGCGCTGACGGAAGCAAAACACCAGCAGCAGTTTTTCCAGTTCCGTTTATCCG
GGCAAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAGCTCCTGCAC
TGGATGGTGGCGCTGGATGGTAAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCC
ACAAGGTAAACAGTTGATTGAACTGCCTGAACTACCGCAGCCGGAGAGCGCCGGGCAACTCT
GGCTCACAGTACGCGTAGTGCAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATCAGC
GCCTGGCAGCAGTGGCGTCTGGCGGAAAACCTCAGTGTGACGCTCCCCGCCGCGTCCCACGC
CATCCCGCATCTGACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGC
AATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATAAAAAACAACTGCTGA
CGCCGCTGCGCGATCAGTTCACCCGTGCACCGCTGGATAACGACATTGGCGTAAGTGAAGCG
ACCCGCATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGA
AGCAGCGTTGTTGCAGTGCACGGCAGATACACTTGCTGATGCGGTGCTGATTACGACCGCTC
ACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGT
AGTGGTCAAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACACCGCATCCGGCGCG
GATTGGCCTGAACTGCCAGCTGGCGCAGGTAGCAGAGCGGGTAAACTGGCTCGGATTAGGGC
CGCAAGAAAACTATCCCGACCGCCTTACTGCCGCCTGTTTTGACCGCTGGGATCTGCCATTGT
CAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCGAA
TTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGCTACAGTCAA
CAGCAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGAAGAAGGCACATGGCTGAAT
ATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCGGA
ATTACAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAATAATAACC
GGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTATGTACTATTTAAAAAACAC
AAACTTTTGGATGTTCGGTTTATTCTTTTTCTTTTACTTTTTTATCATGGGAGCCTACTTCCCGT
TTTTCCCGATTTGGCTACATGACATCAACCATATCAGCAAAAGTGATACGGGTATTATTTTTG
CCGCTATTTCTCTGTTCTCGCTATTATTCCAACCGCTGTTTGGTCTGCTTTCTGACAAACTCGG

FIG. 4 Cont.

CCTCGACTCTAGGCGGCCGCGGGGATCCAGACATGATAAGATACATTGATGAGTTTGGACAA
ACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTA
TTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTT
CAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTCGGATCCTCTAGAGTCGACCTGCAGGCATGC
AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAA
CTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG
CATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCC
TCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAG
GCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAG
GCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
CCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACT
ATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCC
GCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGC
TGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCC
GTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACAC
GACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG
TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTAT
CTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAC
AAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAA
GGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCA
CGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAA
AAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGC
TTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCC
CCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATA
CCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGC
CGAGCGCAGAAGTGGTCCTGCACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
GTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTC
AGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACT
GTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAA
TAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACAT
AGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGAT
CTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATC
TTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGG

FIG. 4 Cont.

GAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCA
TTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAA
TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCA
TGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATG
ACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCT
TAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGC
ACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGC
TGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGG
CCAGT

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08794

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00; C12N 15/09, 5/10, 5/00, 15/63
US CL :800/2; 435/375, 325; 424 93.21, 9.1,9.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/2; 435/375, 325; 424 93.21, 9.1,9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN, BIOSIS, MEDLINE, EMBASE
search terms: transgenic, mmp, gene, reporter, marker, ES, cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KEMP et al. 'The Glycosylation of Alpha-1-Antitrypsin in Transgenic Mice.'Animal Cell Technology: Basic & Applied Aspects, 8th Annual conference. Edited by Funatsu et al. The Netherlands: Kluwer Academic Publishers, 1997, Vol. 8, pages 517-522, see entire article.	1-17
Y	DAMAK et al. Expression of the Human Neutrophil Elastase Gene in the Lungs of Transgenic Mice. Journal of Cellular and Biochemistry Supplement. 03 April 1992, abstract V102, see entire abstract.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 JULY 1998	31 AUG 1998

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIMON et al. Suppressor and activator functions mediated by a repeated heptad sequence in the liver fatty acid-binding protein gene (Fabpl). J. of Biological Chemistry. 18 April 1997, Vol 272, No. 16, pages 10652-10663, see entire article.	1-17
Y	YOSHIZAKI et al. Increased expression of membrane type 1-matrix metalloproteinase in head and neck carcinoma. Cancer. 01 January 1997, Vol. 79, No. 1, pages 139-144, see entire article.	1-17
Y, P	RICE et al. Detection of gelatinase B expression reveals osteoclastic bone resoprtion as a feature of early calvarial bone development. Bone. December 1997, Vol. 21, No. 6, pages 479-486, see entire article.	1-17
Y	HIMELSTEIN et al. Transcriptional activation of the matrix metalloproteinase-9 gene in an H-ras and v-myc transformed rat embryo cell line. Oncogene. 1997, Vol 14, No. 16, pages 1995-1998, see entire article	1-17